US 20140189896A1

(19) **United States**

(12) **Patent Application Publication**

ZHANG et al.

(10) **Pub. No.: US 2014/0189896 A1**

(43) **Pub. Date: Jul. 3, 2014**

(54) **CRISPR-CAS COMPONENT SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION**

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(21) Appl. No.: **14/105,035**

(22) Filed: **Dec. 12, 2013**

Related U.S. Application Data

(60) Provisional application No. 61/835,931, filed on Jun. 17, 2013, provisional application No. 61/791,409, filed on Mar. 15, 2013, provisional application No. 61/768,959, filed on Feb. 25, 2013, provisional application No. 61/748,427, filed on Jan. 2, 2013, provisional application No. 61/736,527, filed on Dec. 12, 2012.

Publication Classification

(51) **Int. Cl.**

C12N 15/85	(2006.01)
C12N 15/74	(2006.01)
C12N 15/70	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/8509*** (2013.01); ***C12N 15/70*** (2013.01); ***C12N 15/85*** (2013.01); ***C12N 15/74*** (2013.01)

USPC .... **800/18**; 435/320.1; 435/252.33; 435/6.18; 435/468; 435/455; 514/44 R; 424/93.21; 435/348; 435/254.2; 435/325

(57) **ABSTRACT**

The invention provides for systems, methods, and compositions for manipulation of sequences and/or activities of target sequences. Provided are vectors and vector systems, some of which encode one or more components of a CRISPR complex, as well as methods for the design and use of such vectors. Also provided are methods of directing CRISPR complex formation in eukaryotic cells and methods for selecting specific cells by introducing precise mutations utilizing the CRISPR/Cas system.

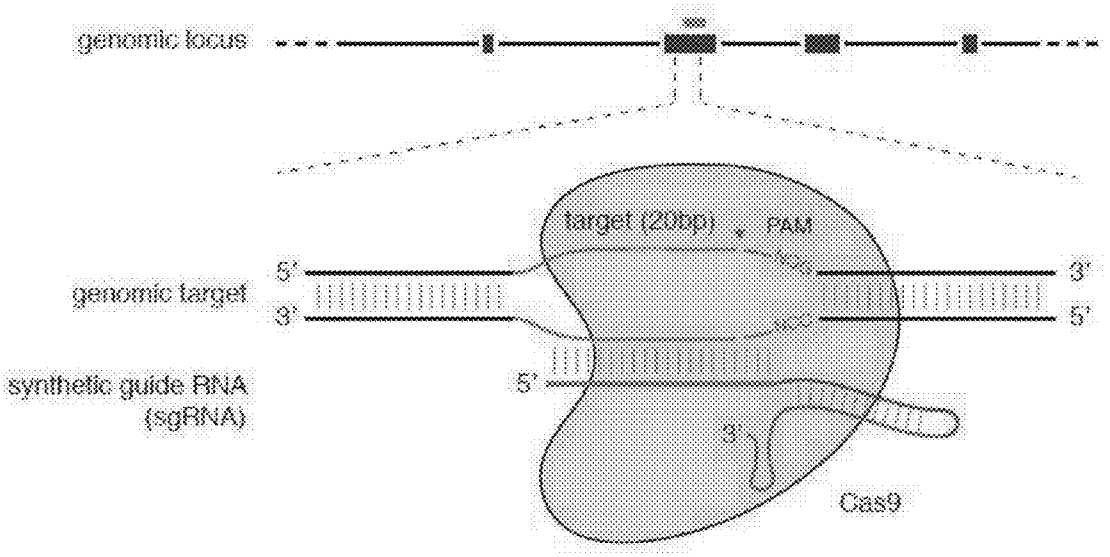

genomic locus
target (20bp)
PAM
5'
3'
genomic target
3'
5'
synthetic guide RNA
(sgRNA)
5'
3'
Cas9

FIG. 1

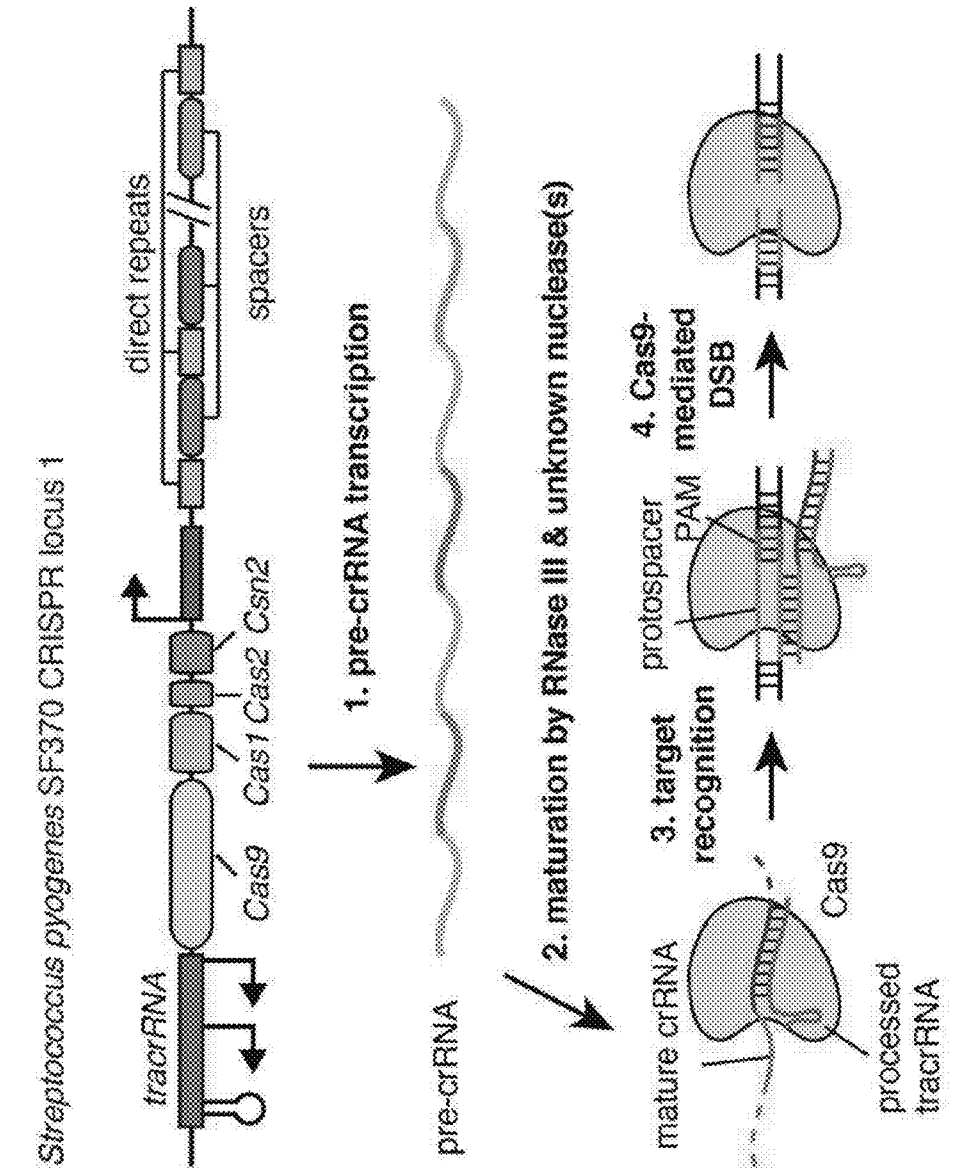

A
Streptococcus pyogenes SF370 CRISPR locus 1
tracrRNA
Cas9
Cas1
Cas2
Csn2
direct repeats
spacers
1. pre-crRNA transcription
pre-crRNA
2. maturation by RNase III & unknown nuclease(s)
mature crRNA
3. target recognition
protospacer
PAM
4. Cas9-mediated DSB
Cas9
processed tracrRNA

FIG. 2A

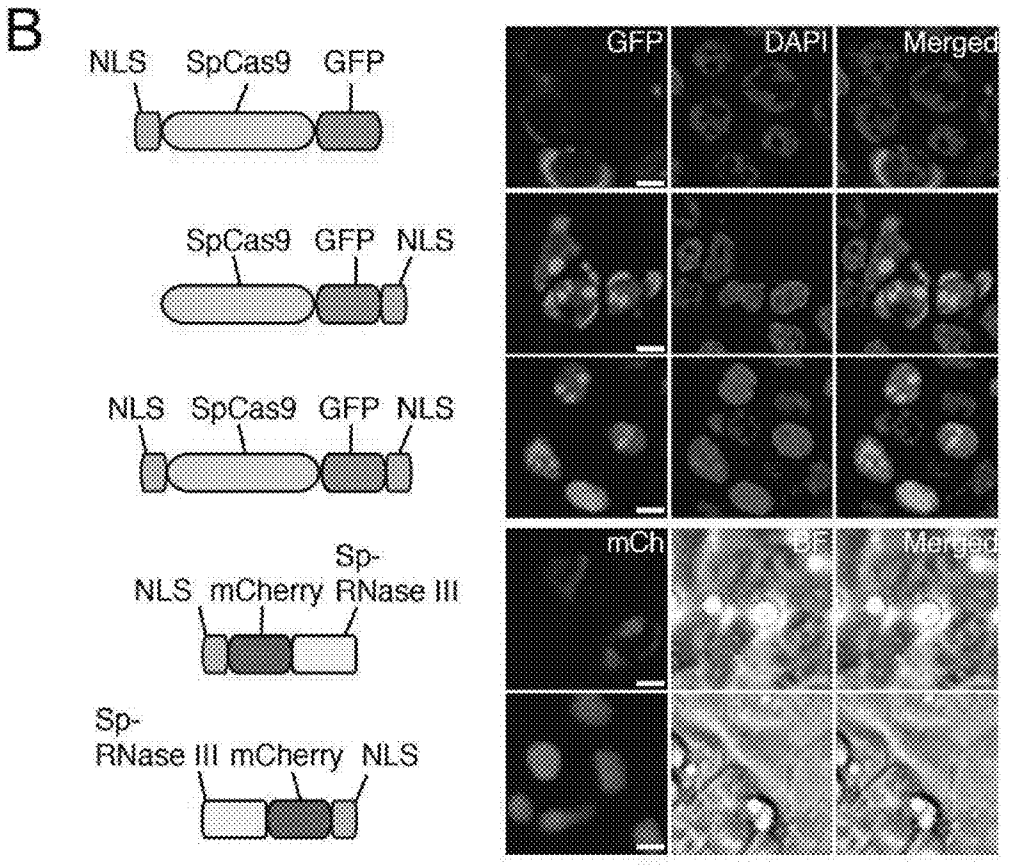

B
NLS SpCas9 GFP
SpCas9 GFP NLS
NLS SpCas9 GFP NLS
Sp-
NLS mCherry RNase III
Sp-
RNase III mCherry NLS
GFP
DAPI
Merged
mCh

FIG. 2B

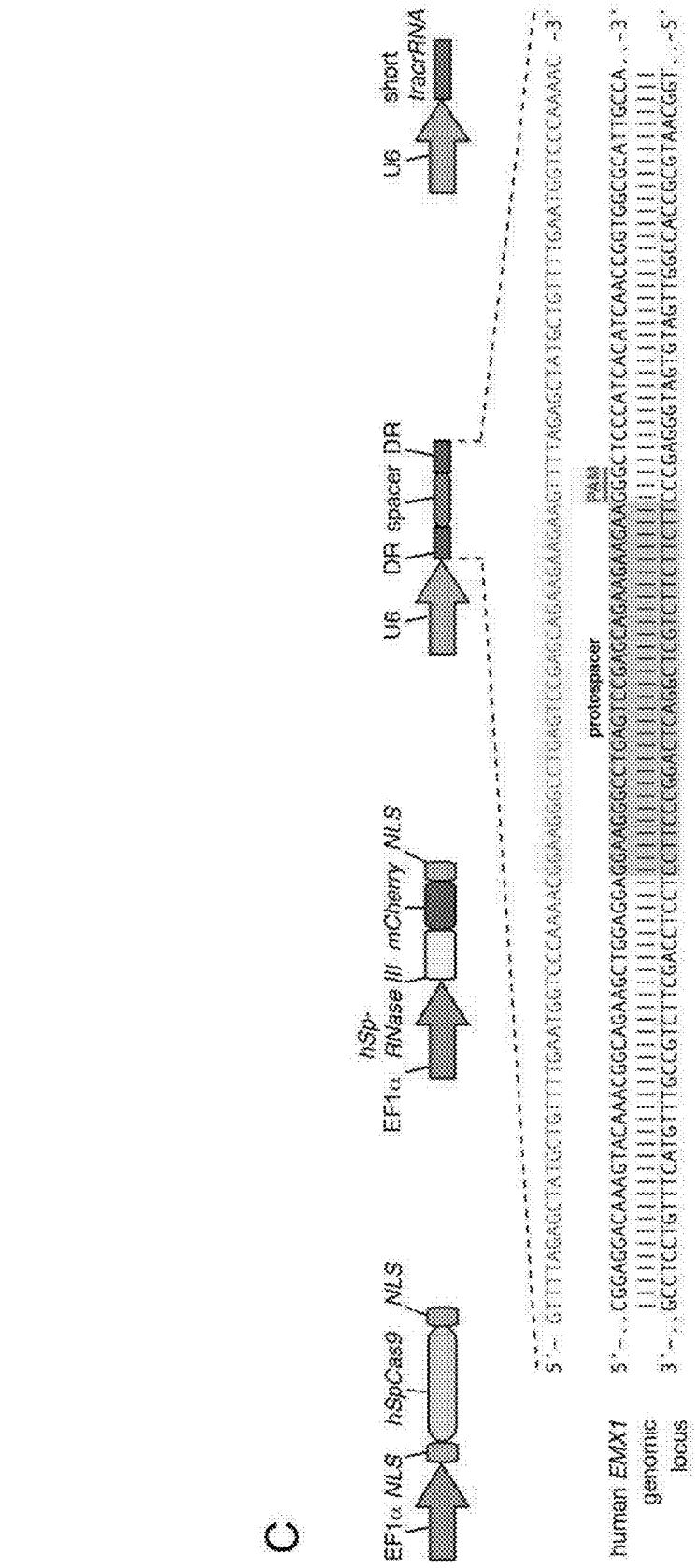

C
EF1α NLS hSpCas9 NLS
EF1α hSp RNase III mCherry NLS
U6 DR spacer DR
U6 short tracrRNA
5'- GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGGGCGGAGGCCTTGACTCAAGAAGAAAGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAAC -3'
protospacer
PAM
human EMX1 genomic locus
5'- ...CGGAGGACAAAGTACAAACGGCAGAAGCTGGAGGAGGAAGAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCA...-3'
3'- ...GCCTCCTGTTTCATGTTTGCCGTCTTCGACCTCCTCCTTCTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTAGTGTAGTTGGCCACCGCGTAACGGT...-5'

FIG. 2C

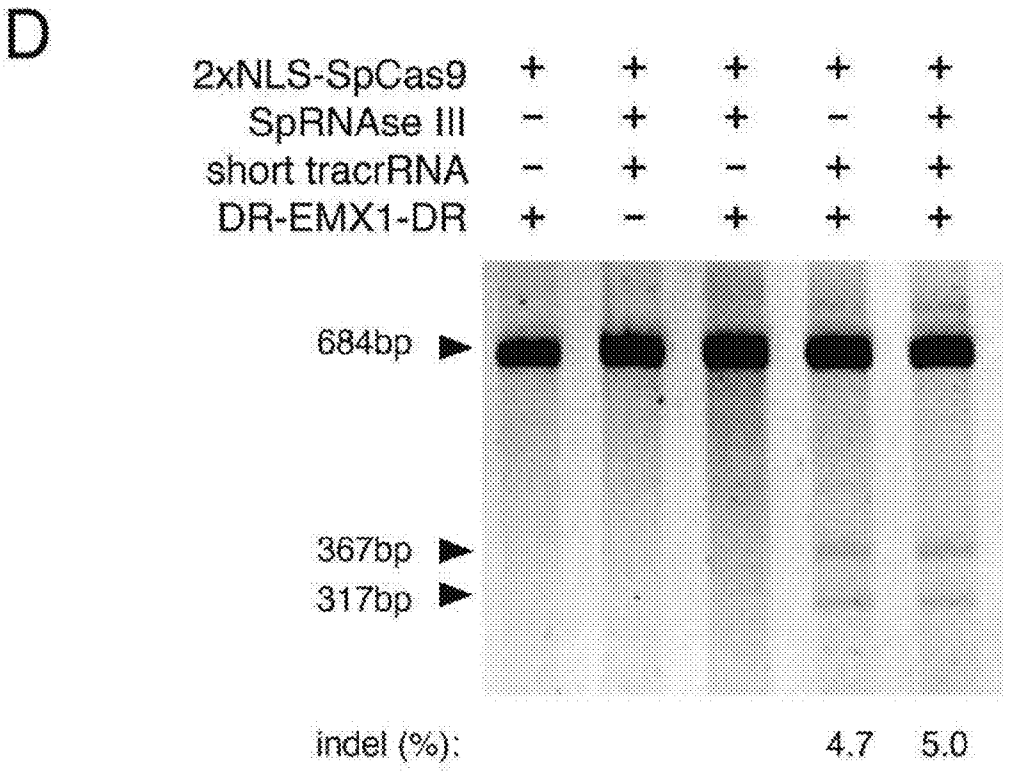
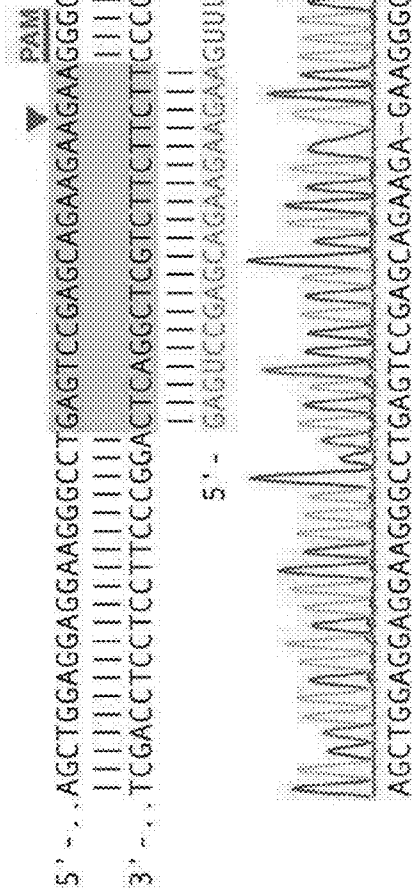

D
2xNLS-SpCas9 + + + + +
SpRNAse III − + + − +
short tracrRNA − + − + +
DR-EMX1-DR + − + + +
684bp
367bp
317bp
indel (%): 4.7 5.0

FIG. 2D


E
Target locus
PAM
5' ~ . . AGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAC . . -3'
3' ~ . . TCGACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTG . . -5'
crRNA
5' - GAGUCCGAGCAGAAGAAGUUUUAGAGC . . -3'
indel
AGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGA-GAAGGGCTCCCAT

F
human EMX1 protospacer target (mutation in 5 of 43 sequenced clones = 11.6%)
WT 5' ~ . . CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAG AAGAAGGGCTCCCATCACAT . . -3'
Δ1 CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAG---AGAAGGGCTCCCATCACAT
+1 CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACAT
Δ3 CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAG----AAGGGCTCCCATCACAT
m1, Δ6 CTGGAGGAGGAAGGGCCTGAGCCCGAGCAGAAG--------GGCTCCCATCACAT

FIG. 2E-F

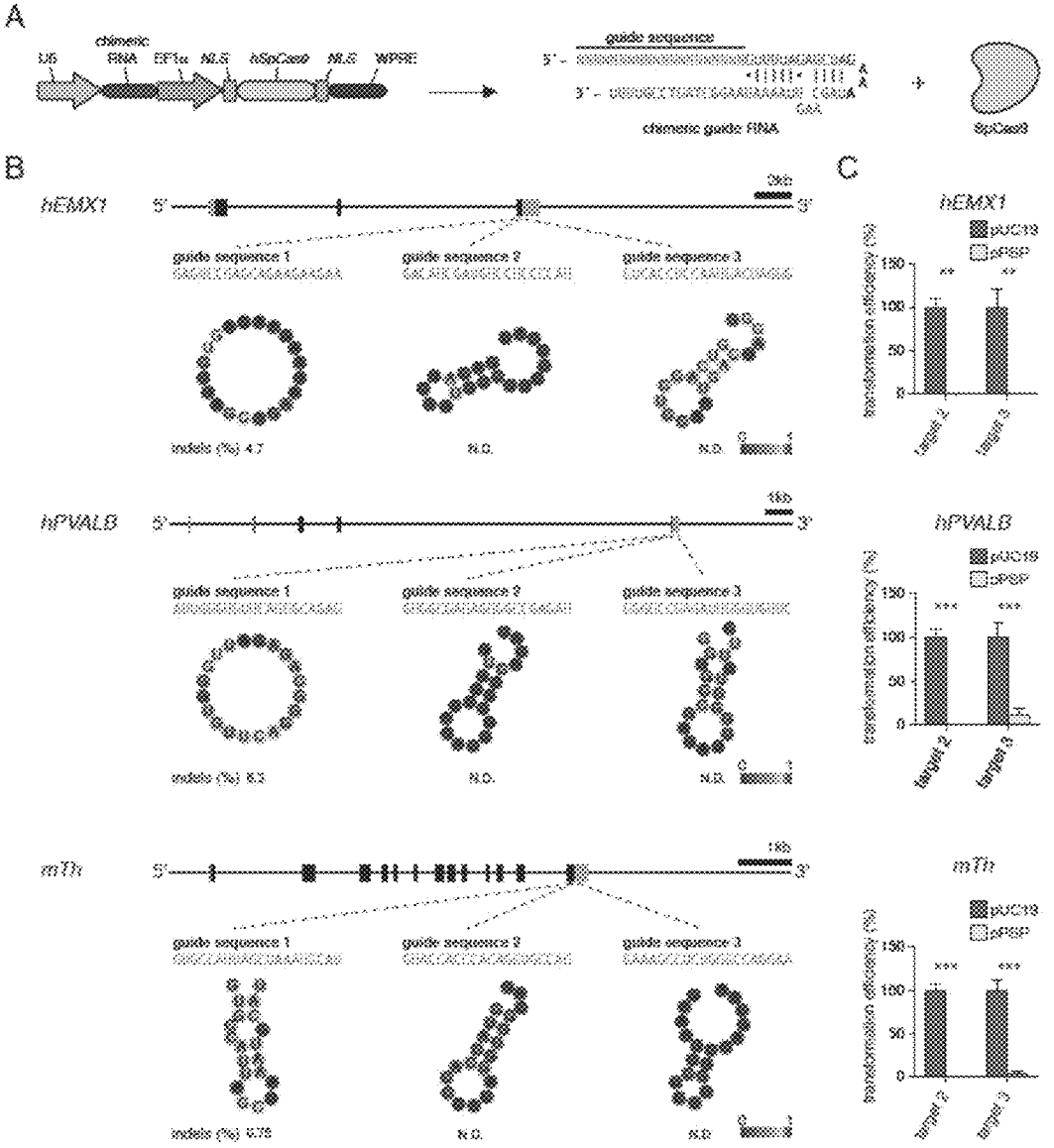

A
U6
chimeric RNA
EF1α
NLS
hSpCas9
NLS
WPRE
guide sequence
chimeric guide RNA
SpCas9
B
hEMX1
hPVALB
mTh
guide sequence 1
guide sequence 2
guide sequence 3
indels (%) 4.7
indels (%) 8.5
indels (%) 6.76
N.D.
C
hEMX1
hPVALB
mTh
pUC19
sPSP
transformation efficiency (%)
target 2
target 3

FIG. 3

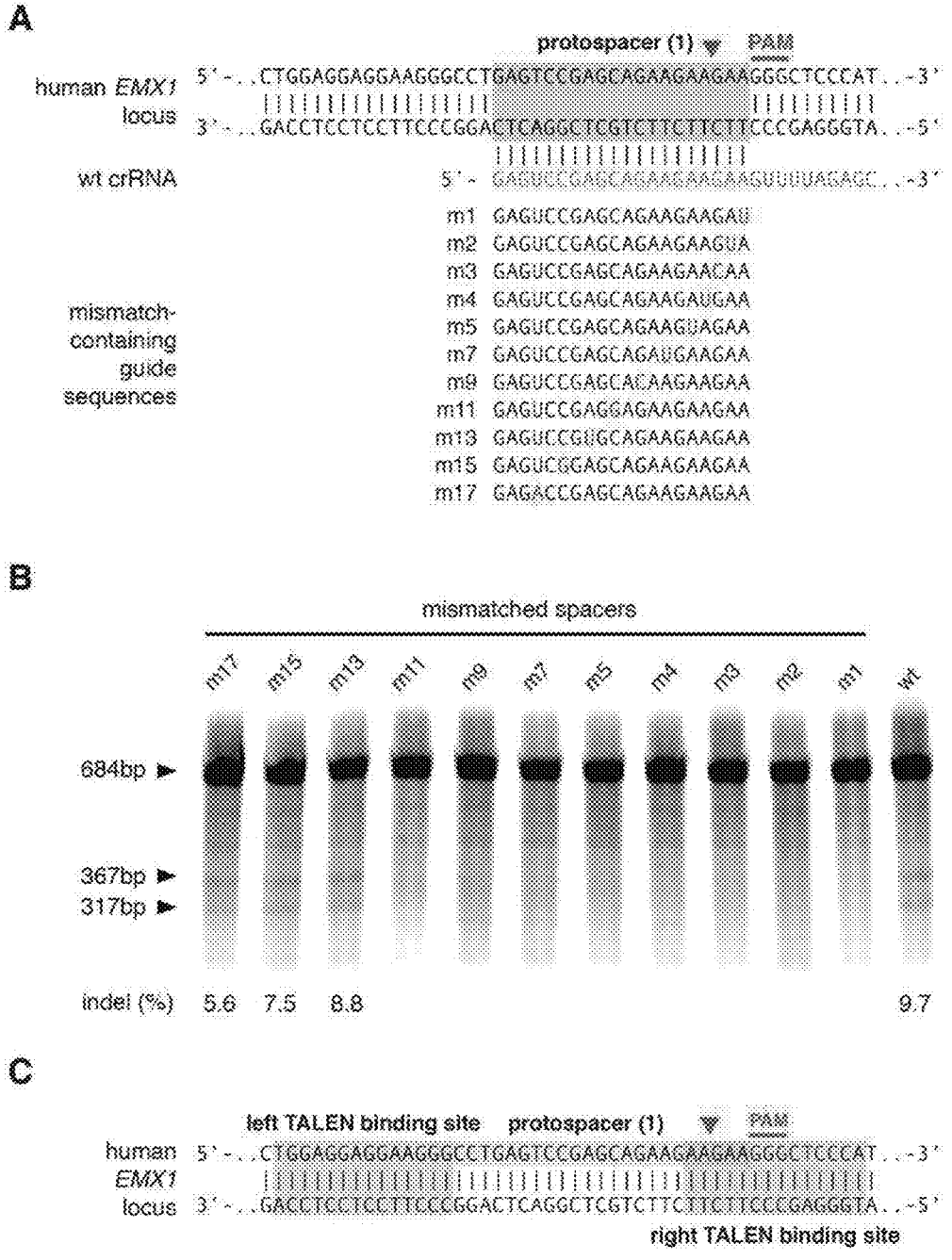

A
protospacer (1)
PAM
human EMX1 locus
5'-..CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAT..-3'
3'-..GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTA..-5'
wt crRNA
5'- GAGUCCGAGCAGAAGAAGAAGUUUUAGAGC..-3'
mismatch-containing guide sequences
m1 GAGUCCGAGCAGAAGAAGAU
m2 GAGUCCGAGCAGAAGAAGUA
m3 GAGUCCGAGCAGAAGAACAA
m4 GAGUCCGAGCAGAAGAUGAA
m5 GAGUCCGAGCAGAAGUAGAA
m7 GAGUCCGAGCAGAUGAAGAA
m9 GAGUCCGAGCACAAGAAGAA
m11 GAGUCCGAGGAGAAGAAGAA
m13 GAGUCCGUGCAGAAGAAGAA
m15 GAGUCGGAGCAGAAGAAGAA
m17 GAGACCGAGCAGAAGAAGAA
B
mismatched spacers
m17
m15
m13
m11
m9
m7
m5
m4
m3
m2
m1
wt
684bp
367bp
317bp
indel (%)
5.6
7.5
8.8
9.7
C
left TALEN binding site
protospacer (1)
PAM
human EMX1 locus
5'-..CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAT..-3'
3'-..GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTA..-5'
right TALEN binding site

FIG. 4A-C

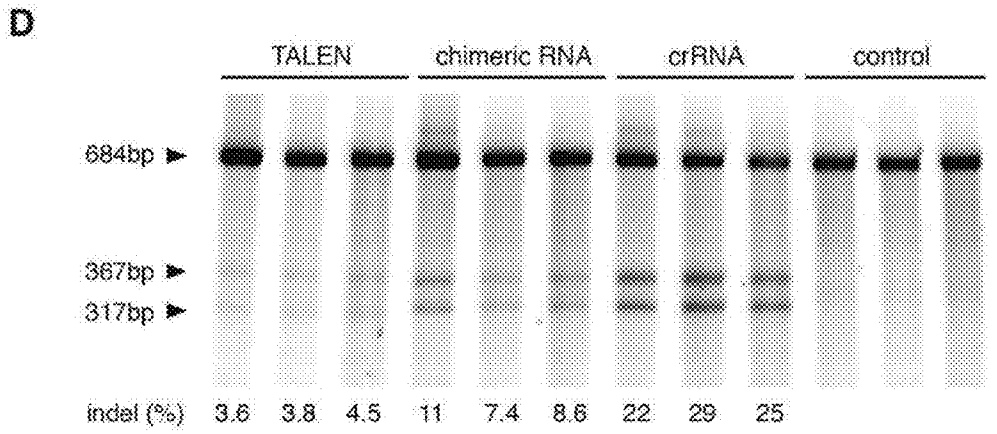

D
TALEN
chimeric RNA
crRNA
control
684bp
367bp
317bp
indel (%)
3.6
3.8
4.5
11
7.4
8.6
22
29
25

FIG. 4D

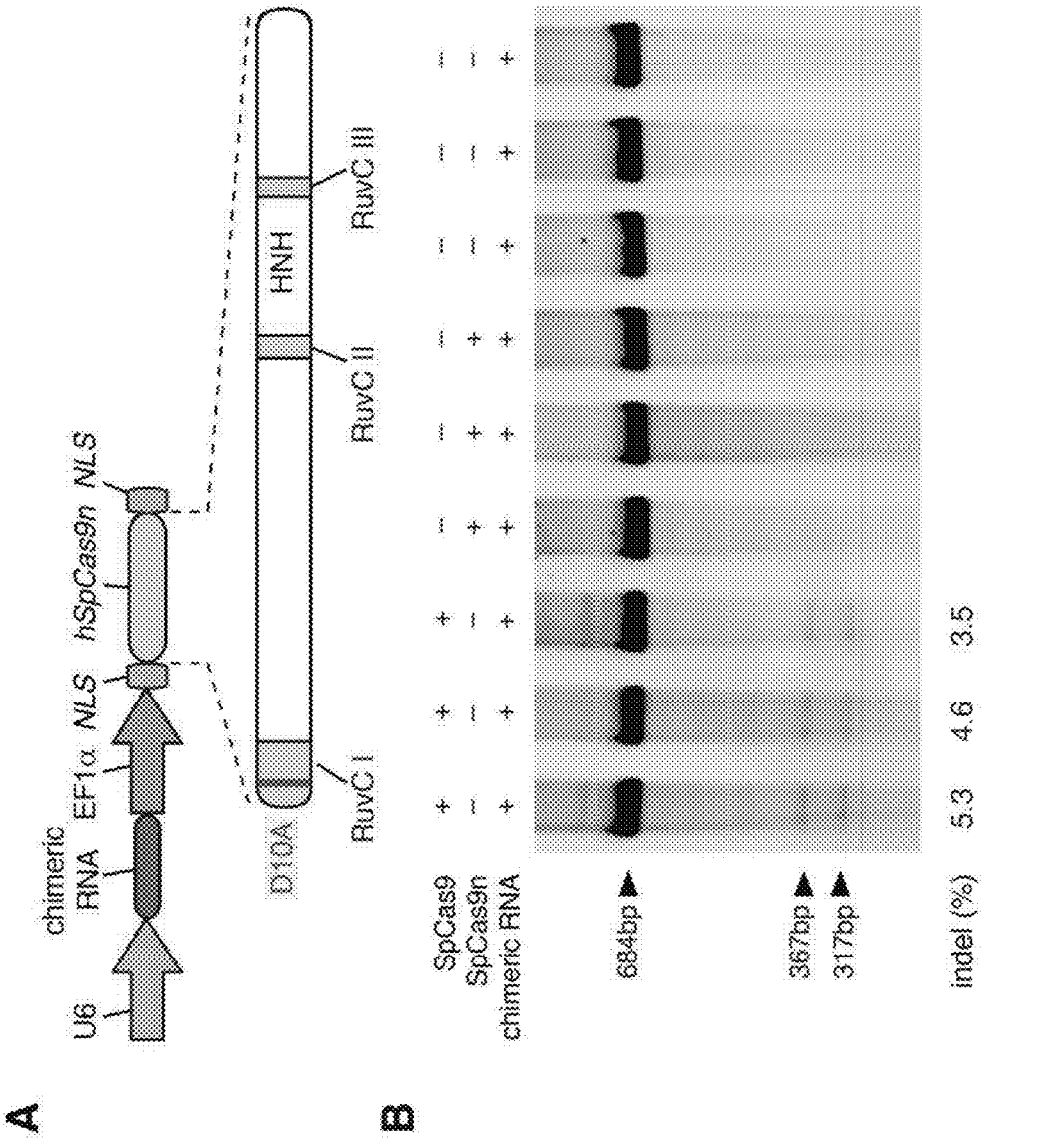

A
U6
chimeric RNA
EF1α
NLS
hSpCas9n
NLS
D10A
RuvC I
RuvC II
HNH
RuvC III
B
SpCas9
SpCas9n
chimeric RNA
684bp
367bp
317bp
indel (%)
5.3
4.6
3.5

FIG. 5A-B

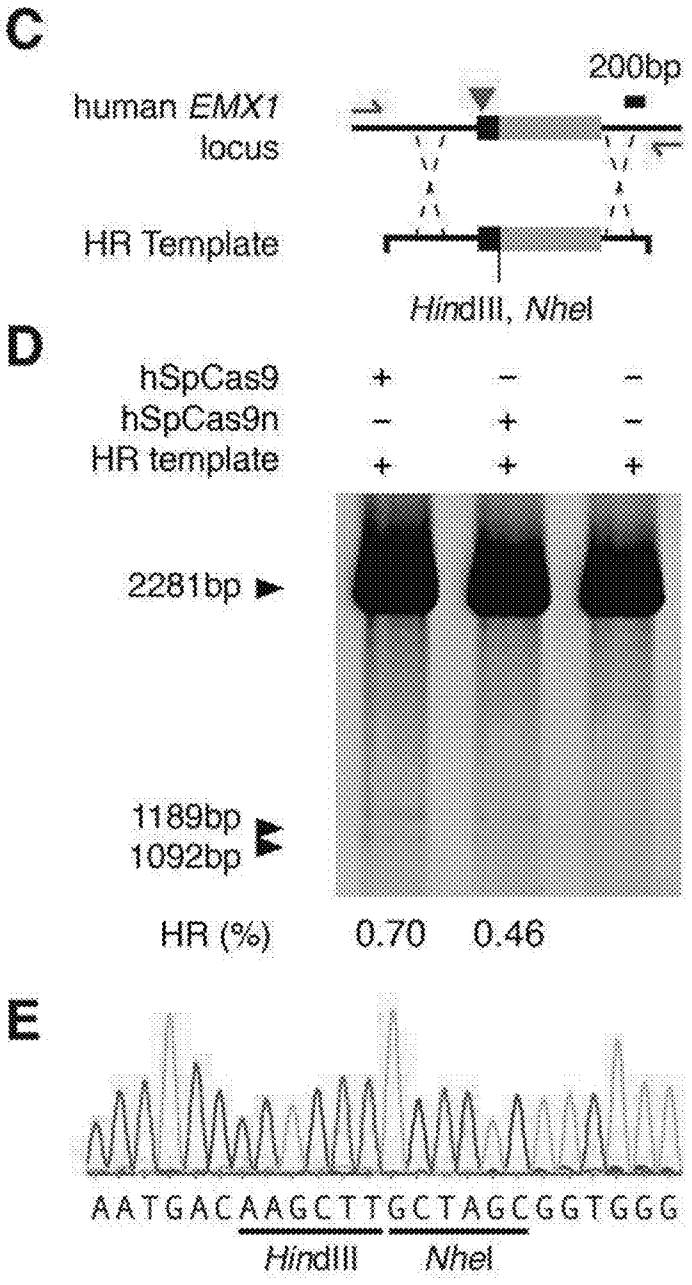

C
200bp
human EMX1 locus
HR Template
HindIII, NheI
D
hSpCas9
hSpCas9n
HR template
+ − −
− + −
+ + +
2281bp
1189bp
1092bp
HR (%)
0.70 0.46
E
AATGACAAGCTTGCTAGCGGTGGG
HindIII
NheI

FIG. 5C-E

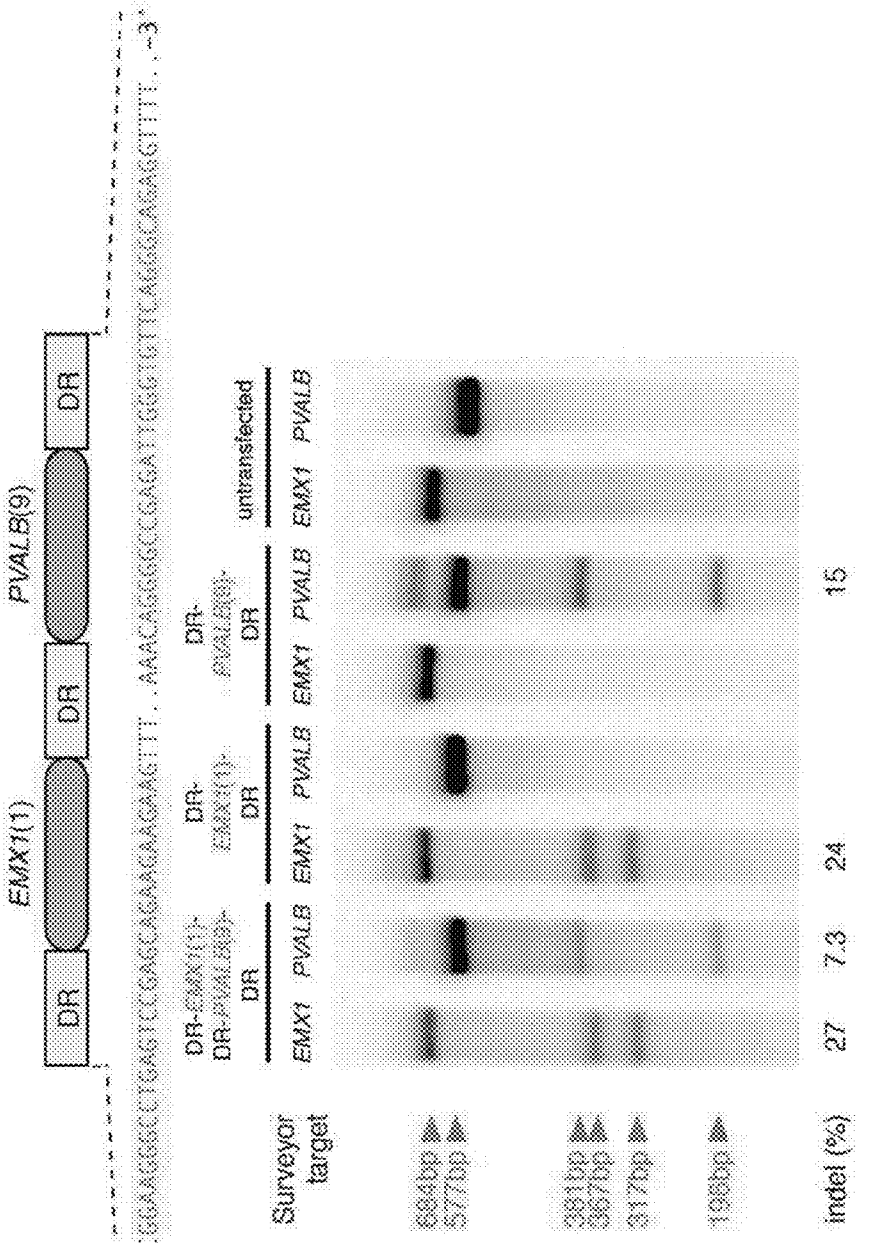

F
crRNA array design
EMX1(1)
PVALB(9)
DR
DR
DR
DR
5'-...AAACGUAGGGCCUGAGUCCGAGCAGAAGAAGAAGUUUU...AAACAGGGCCGAGAUUGGGUGUUCAGAGCAAGGUUUU...-3'
Surveyor target
DR-EMX1(1)-DR-PVALB(9)-DR
DR-EMX1(1)-DR
DR-PVALB(9)-DR
untransfected
EMX1
PVALB
EMX1
PVALB
EMX1
PVALB
EMX1
PVALB
694bp
577bp
391bp
367bp
317bp
198bp
indel (%)
27
7.3
24
15

FIG. 5F

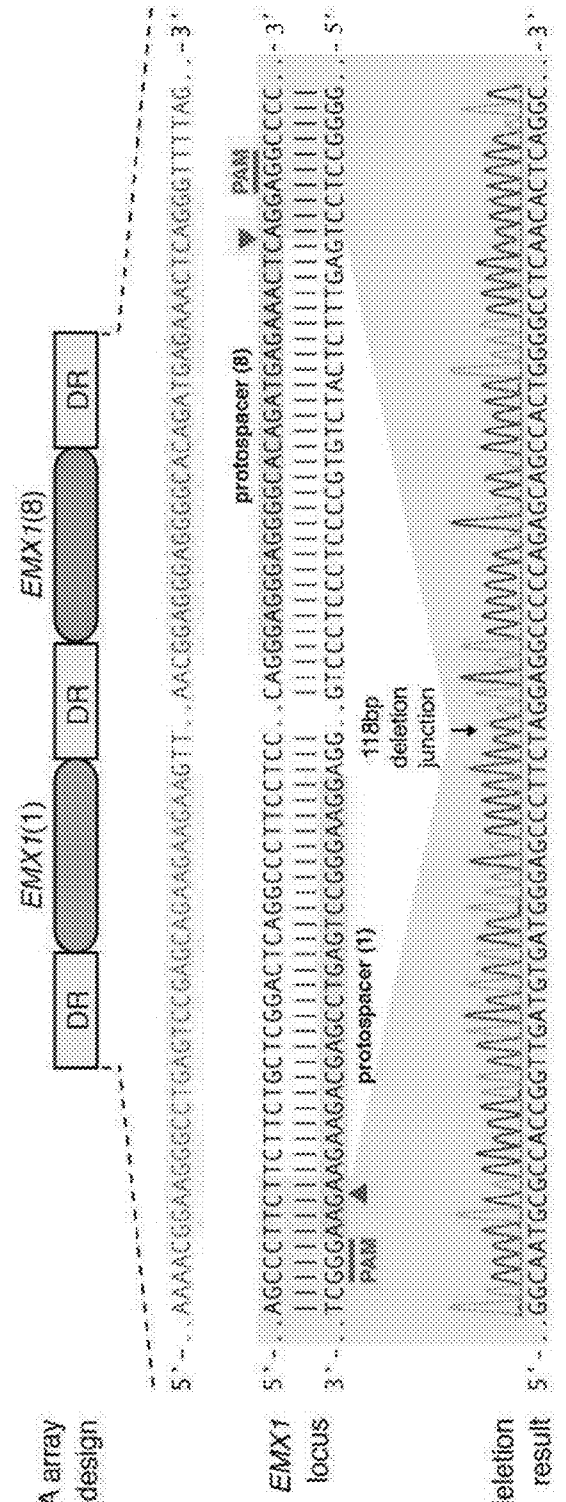
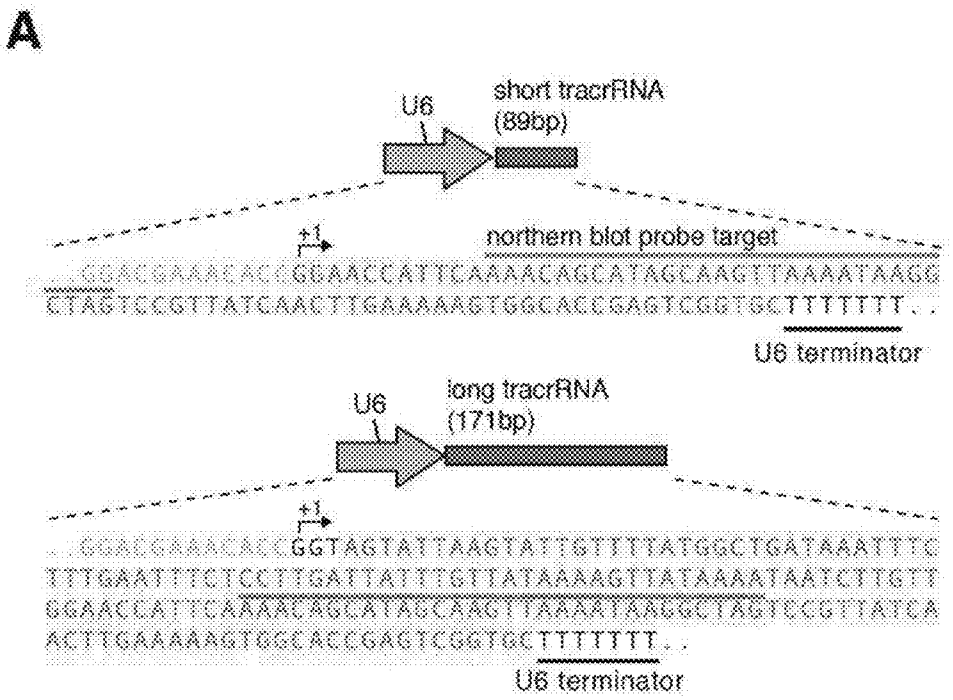

G
crRNA array design
DR
EMX1(1)
DR
EMX1(8)
DR
5' - ...AAAACGGAAGGGGCCTGAGTCCGAGCAGAAGAAGAAGTT...AACGGAGGGAGGGGCACAGATGAGAAACTCAGGGTTTTAG...-3'
human EMX1 locus
5' - ...AGCCCTTCTTCTTCTGCTCGGACTCAGGCCCTTCCTCC...CAGGGAGGGAGGGGCACAGATGAGAAACTCAGGAGGCCCC...-3'
3' - ...TCGGGAAGAAGACGACGAGCCTGAGTCCGGGAAGGAGG...GTCCCTCCCTCCCCGTGTCTACTCTTTGAGTCCTCCGGGG...-5'
protospacer (1)
PAM
118bp deletion junction
protospacer (8)
PAM
deletion result
5' - ...GGCAATGCGCCACCGGTTGATGTGATGGGAGCCCTTCTAGGAGGCCCCCAGAGCAGCCACTGGGGCCTCAACACTCAGGC...-3'

FIG. 5G

Cas9	target species	gene	protospacer ID	protospacer sequence (5' to 3')	PAM	strand	cell line tested	% indel (pre-crRNA + tracrRNA)	% indel (chimeric RNA)
S. pyogenes SF370 type II CRISPR	*Homo sapiens*	*EMX1*	1	GGAAGGGCCTGAGTCCGAGCAGAAGAAGAA	GGG	+	293FT	20 ± 1.8	6.7 ± 0.62
		EMX1	2	CATTGGAGGTGACATCGATGTCCTCCCCAT	TGG	–	293FT	2.1 ± 0.31	N.D.
		EMX1	3	GGACATCGATGTCACCTCCAATGACTAGGG	TGG	+	293FT	14 ± 1.1	N.D.
		EMX1	4	CATCGATGTCCTCCCCATTGGCCTGCTTCG	TGG	–	293FT	11 ± 1.7	N.D.
		EMX1	5	TTCGTGGCAATGCGCCACCGGTTGATGTGA	TGG	–	293FT	4.3 ± 0.46	2.1 ± 0.51
		EMX1	6	TCGTGGCAATGCGCCACCGGTTGATGTGAT	GGG	–	293FT	4.0 ± 0.68	0.41 ± 0.25
		EMX1	7	TCCAGCTTCTGCCGTTTGTACTTTGTCCTC	CGG	–	293FT	1.5 ± 0.12	N.D.
		EMX1	8	GGAGGGAGGGGCACACATGACAAACTCAGG	AGG	–	293FT	7.8 ± 0.83	2.3 ± 1.2
	Homo sapiens	*PVALB*	9	AGGGGCCGAGATTGGGTGTTCAGGGCAGAG	AGG	+	293FT	21 ± 2.6	6.5 ± 0.32
		PVALB	10	ATGCAGGAGGGTGGCGAGAGGGGCCGAGAT	TGG	+	293FT	N.D.	N.D.
		PVALB	11	GGTGGCGAGAGGGGCCGAGATTGGGTGTTC	AGG	+	293FT	N.D.	N.D.
	Mus musculus	*Th*	12	CAAGCACTGAGTGCCATTAGCTAAATGCAT	AGG	–	Neuro2A	27 ± 4.3	4.1 ± 2.2
		Th	13	AATGCATAGGGTACCACCCACAGGTGCCAG	GGG	–	Neuro2A	4.8 ± 1.2	N.D.
		Th	14	ACACACATGGGAAAGCCTCTGGGCCAGGAA	AGG	+	Neuro2A	11.3 ± 1.3	N.D.
S. thermophilus LMD-9 CRISPR1	*Homo sapiens*	*EMX1*	15	GGAGGAGGTAGTATACAGAAACACAGAGAA	GTAGAAT	–	293FT	14 ± 0.88	N.T.
		EMX1	16	AGAATGTAGAGGAGTCACAGAAACTCAGCA	CTAGAAA	–	293FT	7.8 ± 0.77	N.T.

FIG. 6


A
U6
short tracrRNA
(89bp)
+1
northern blot probe target
GGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGG
CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT..
U6 terminator
U6
long tracrRNA
(171bp)
+1
GGTAGTATTAAGTATTGTTTTATGGCTGATAAATTTC
TTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTT
GGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT..
U6 terminator

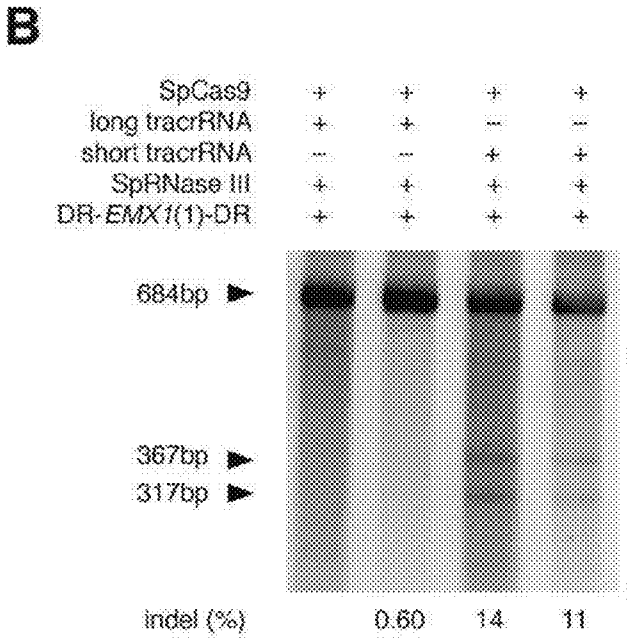

B
SpCas9 + + + +
long tracrRNA + + – –
short tracrRNA – – + +
SpRNase III + + + +
DR-EMX1(1)-DR + + + +
684bp
367bp
317bp
indel (%) 0.60 14 11

FIG. 7A-B

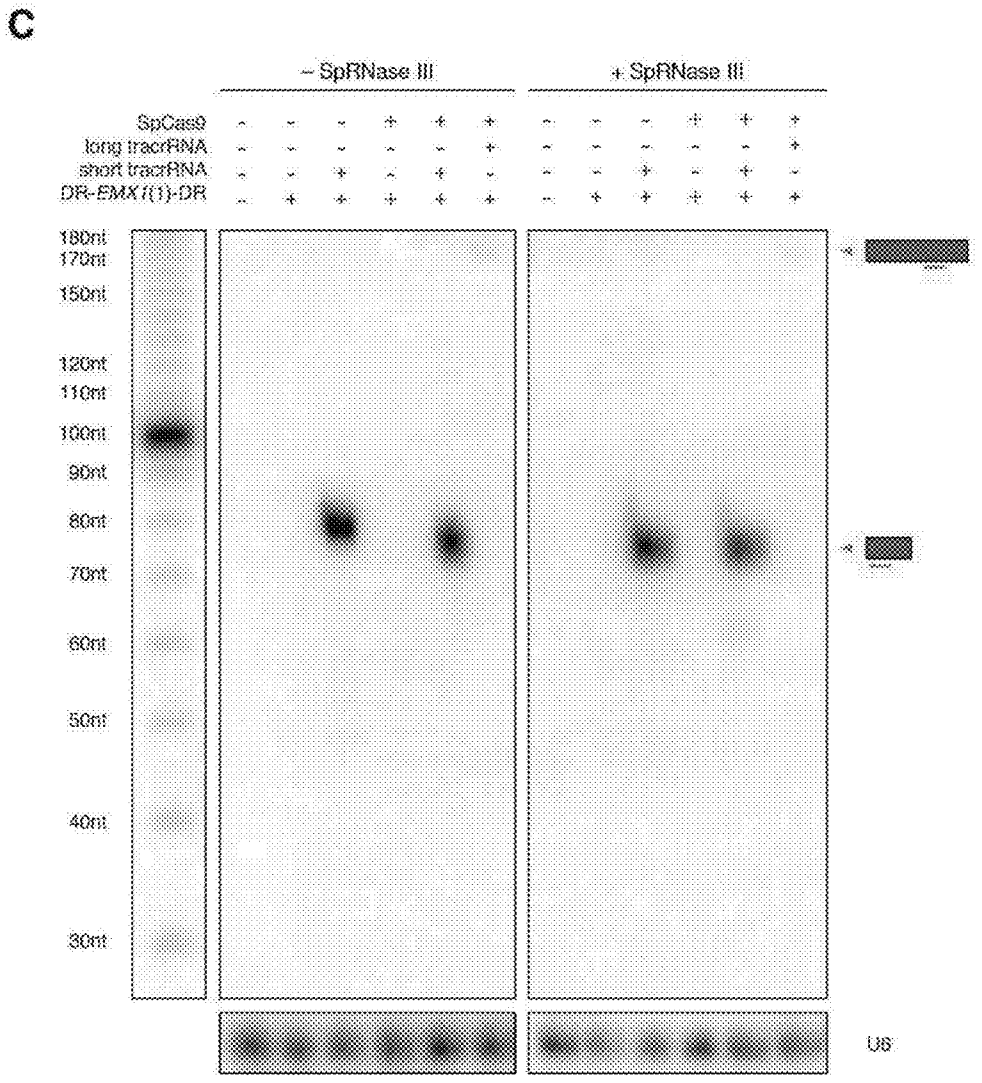

C
– SpRNase III
+ SpRNase III
SpCas9
long tracrRNA
short tracrRNA
DR-EMX1(1)-DR
180nt
170nt
150nt
120nt
110nt
100nt
90nt
80nt
70nt
60nt
50nt
40nt
30nt
U6

FIG. 7C

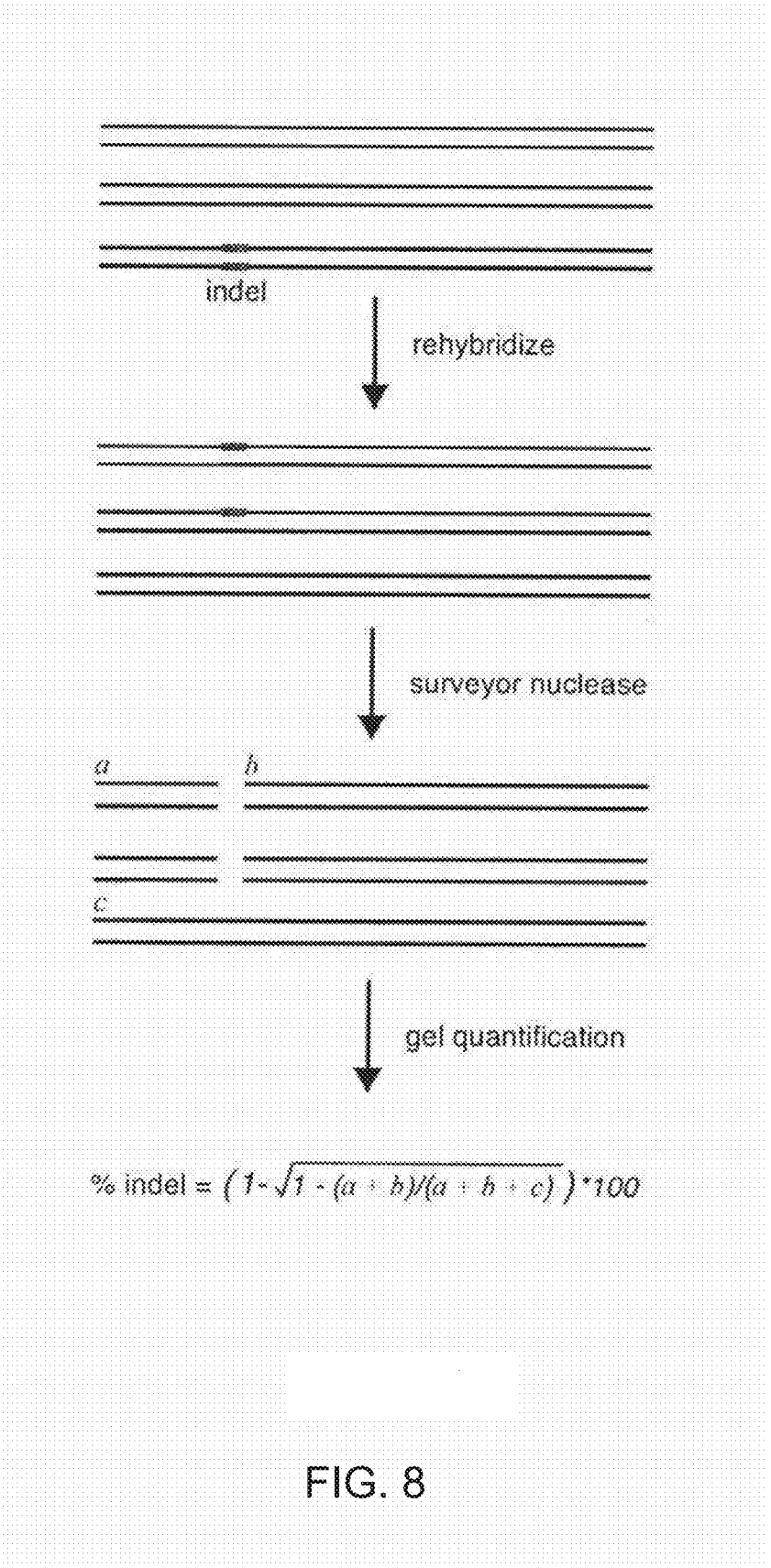

indel
rehybridize
surveyor nuclease
a
b
c
gel quantification
% indel = (1-√1 - (a + b)/(a + b + c)) *100

FIG. 8

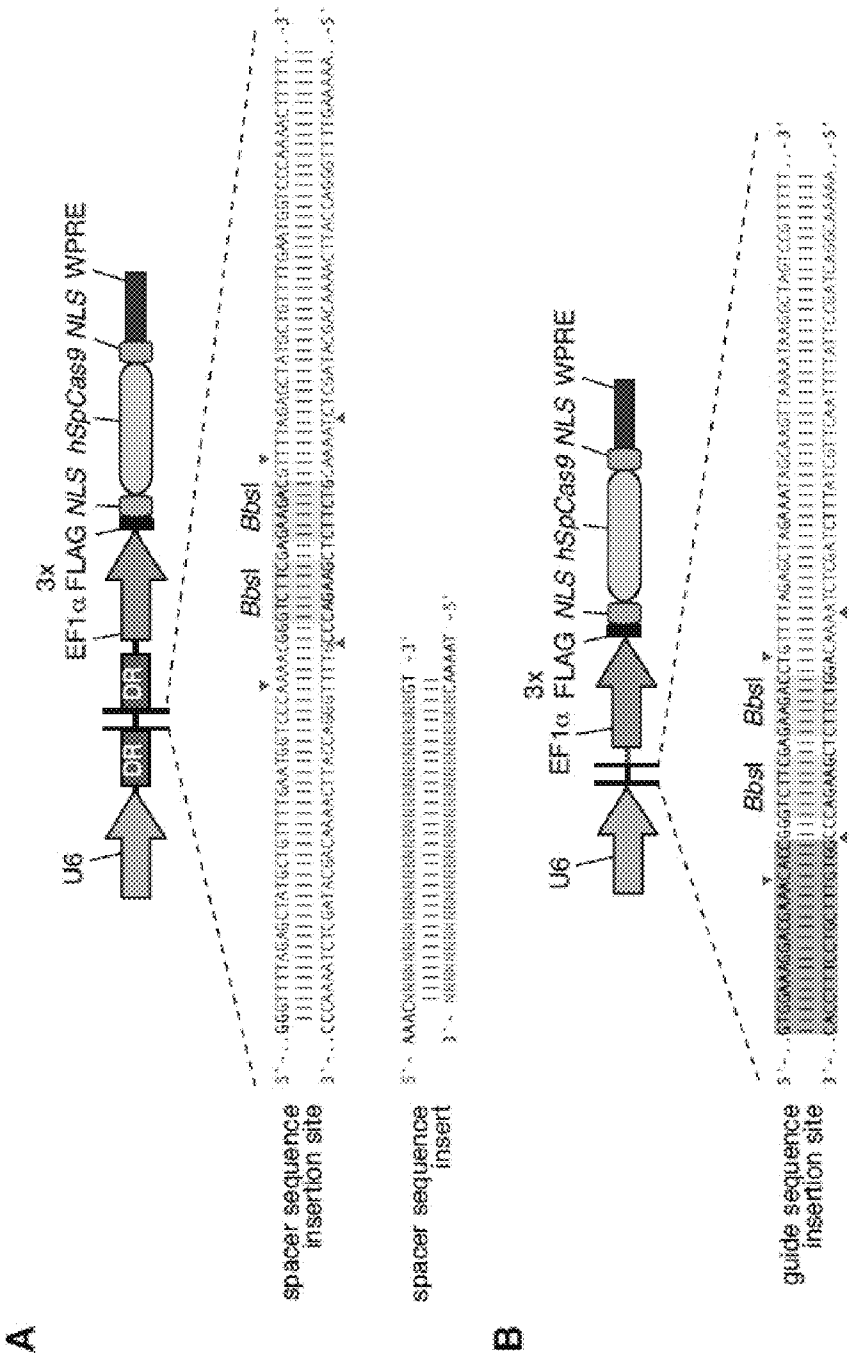

A
U6
BsI
BsI
3x
EF1α FLAG NLS hSpCas9 NLS WPRE
BbsI
BbsI
spacer sequence insertion site
spacer sequence insert
B
U6
3x
EF1α FLAG NLS hSpCas9 NLS WPRE
BbsI
BbsI
guide sequence insertion site
guide sequence insert

FIG. 9A-B

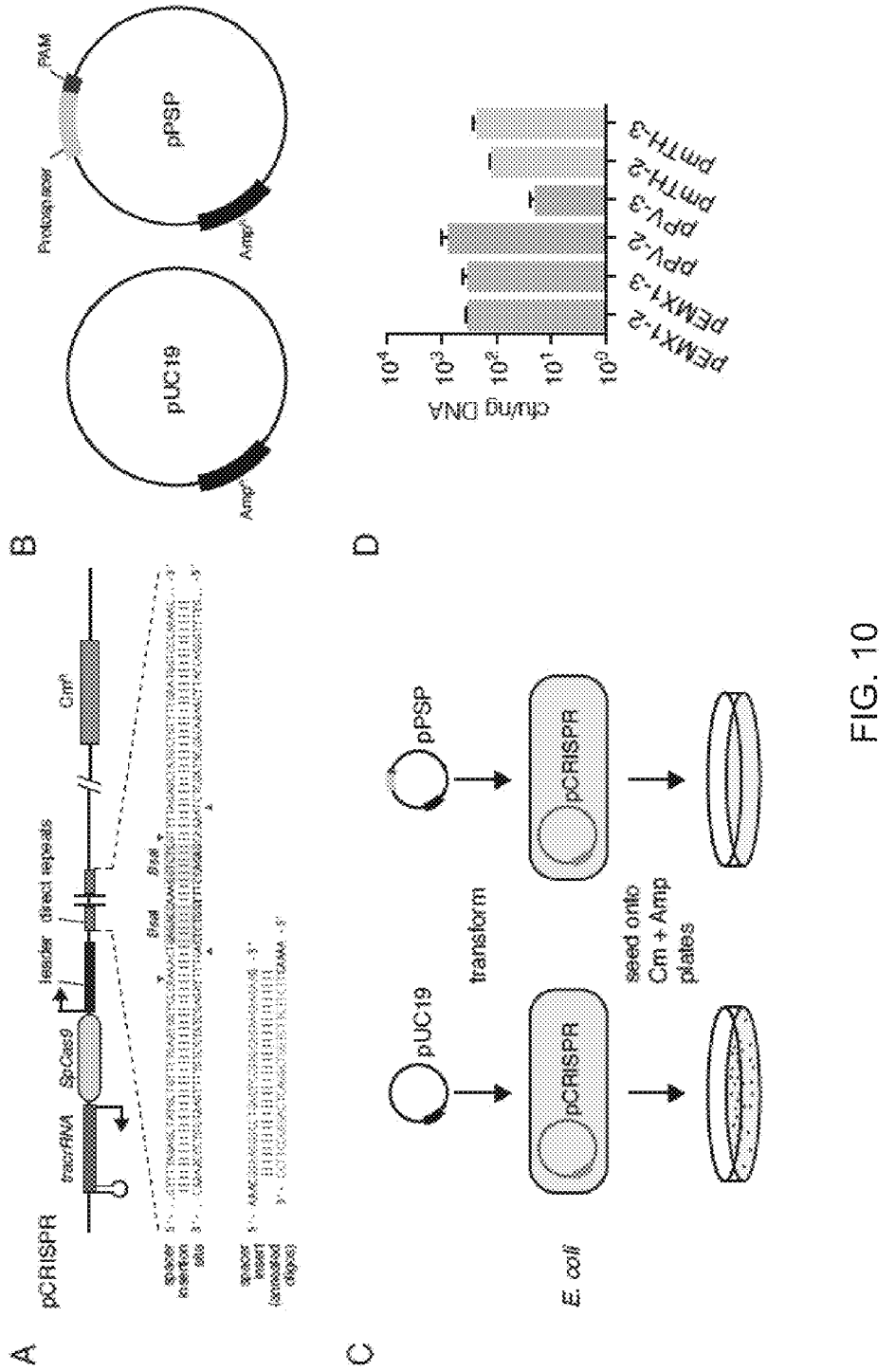
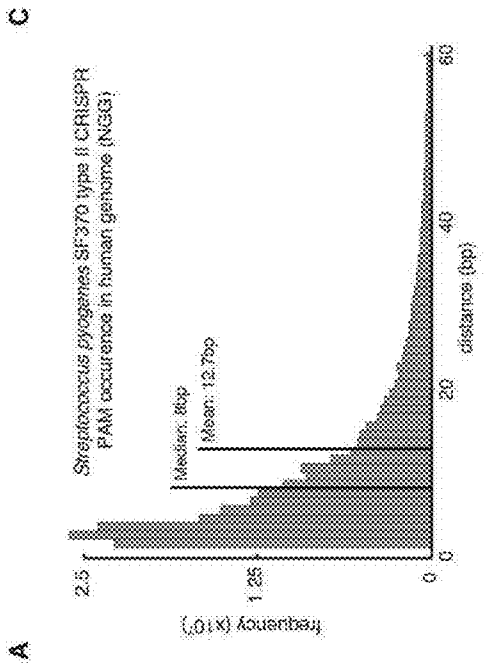

A
pCRISPR
tracrRNA
SpCas9
leader
direct repeats
Cm^R
B
pUC19
pPSP
Amp^R
Protospacer
PAM
D
cfu/ng DNA
pEMX1-2
pEMX1-3
pPLV-2
pPLV-3
pMTH-2
pMTH-3
C
E. coli
pUC19
pPSP
transform
pCRISPR
seed onto Cm + Amp plates

FIG. 10



C

	NGG		NNAGAAW	
Chr	median	mean	median	mean
1	7	12.8	67	115.8
2	8	12.7	64	100.8
3	8	13.0	63	98.5
4	9	14.0	61	94.5
5	8	13.1	63	97.9
6	8	13.1	63	98.5
7	8	12.4	64	102.9
8	8	12.8	64	100.9
9	7	13.9	65	120.5
10	7	12.1	66	107.0
11	7	12.0	65	105.8
12	8	12.4	65	103.5
13	8	13.6	62	94.6
14	8	12.0	65	101.5
15	7	11.5	68	107.7
16	7	11.7	74	136.8
17	6	10.3	76	127.9
18	8	13.4	63	101.8
19	6	9.4	82	145.4
20	7	11.1	72	121.8
21	7	13.4	64	111.4
22	6	9.2	85	140.3
X	8	13.2	63	99.0
Y	8	29.2	62	223.7

FIG. 11A-C

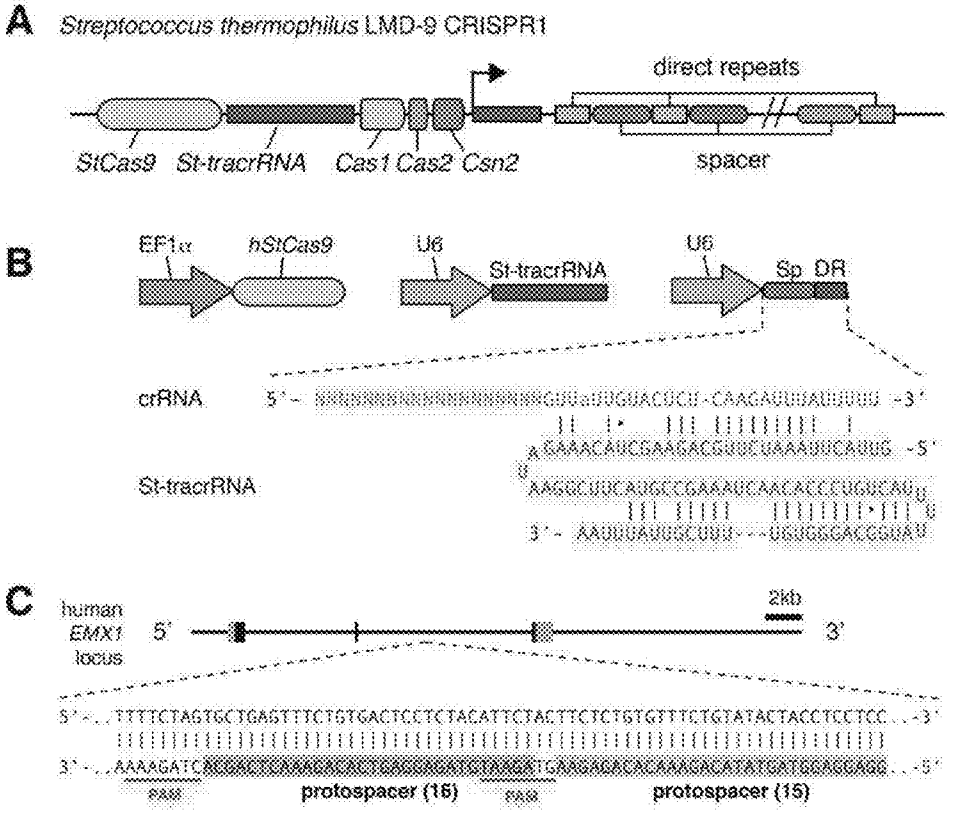

A
Streptococcus thermophilus LMD-9 CRISPR1
direct repeats
StCas9
St-tracrRNA
Cas1
Cas2
Csn2
spacer
B
EF1α
hStCas9
U6
St-tracrRNA
U6
Sp DR
crRNA
St-tracrRNA
C
human
EMX1
locus
5'
2kb
3'
PAM
protospacer (16)
PAM
protospacer (15)

FIG. 12A-C

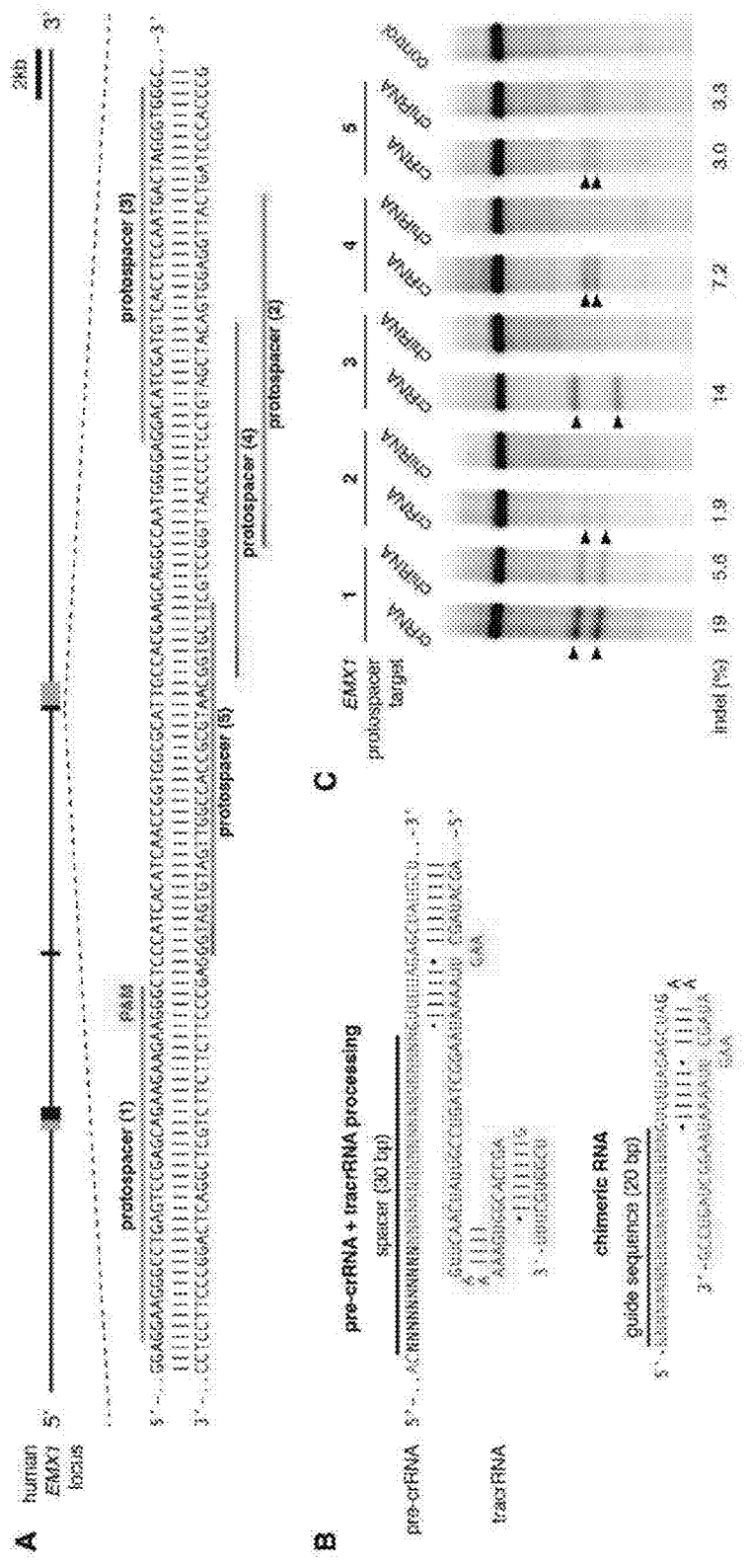

A
human EMX1 locus
5'
3'
2kb
protospacer (1)
PAM
protospacer (3)
protospacer (5)
protospacer (4)
protospacer (2)
B
pre-crRNA + tracrRNA processing
spacer (30 bp)
pre-crRNA
tracrRNA
chimeric RNA
guide sequence (20 bp)
C
EMX1 protospacer target
crRNA
chiRNA
indel (%)
19
5.6
1.9
14
7.2
3.0
3.3
control

FIG. 13A-C

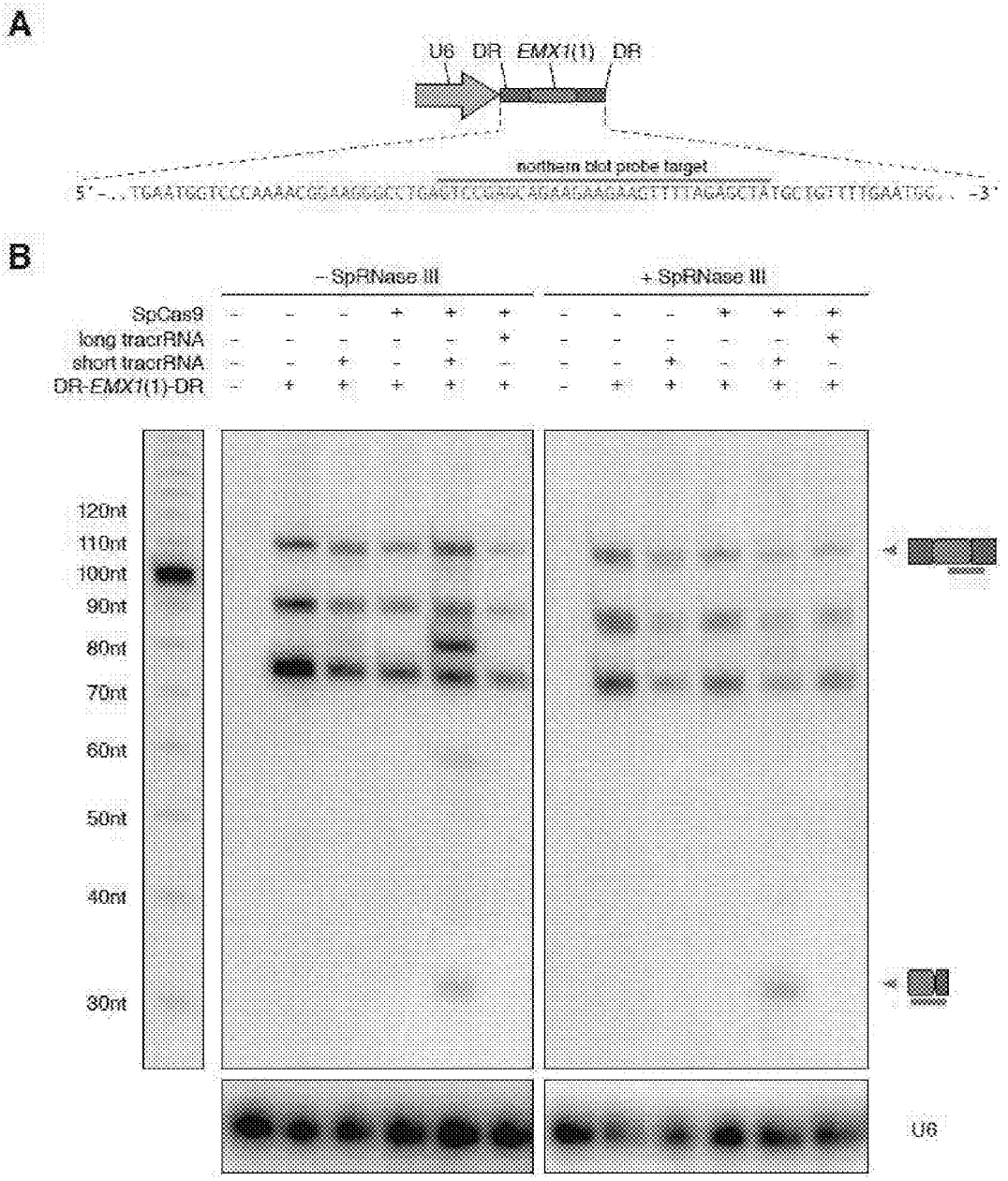

A
U6
DR
EMX1(1)
DR
northern blot probe target
B
– SpRNase III
+ SpRNase III
SpCas9
long tracrRNA
short tracrRNA
DR-EMX1(1)-DR
120nt
110nt
100nt
90nt
80nt
70nt
60nt
50nt
40nt
30nt
U6

FIG. 14A-B

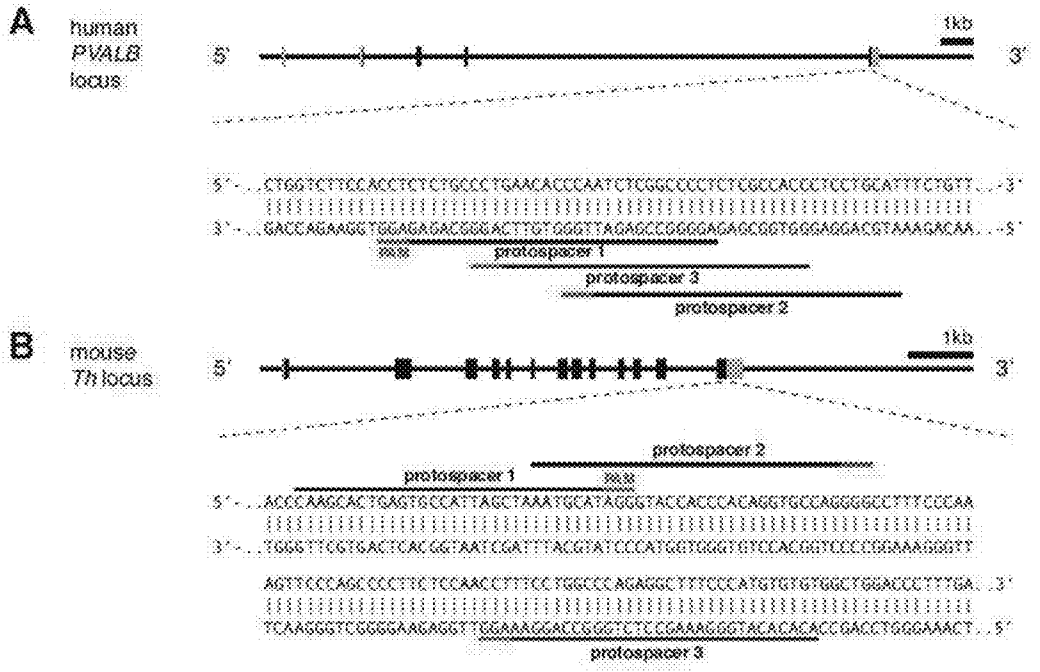

A
human
PVALB
locus
1kb
5'
3'
protospacer 1
protospacer 3
protospacer 2
B
mouse
Th locus
1kb
5'
3'
protospacer 2
protospacer 1
protospacer 3

FIG. 15

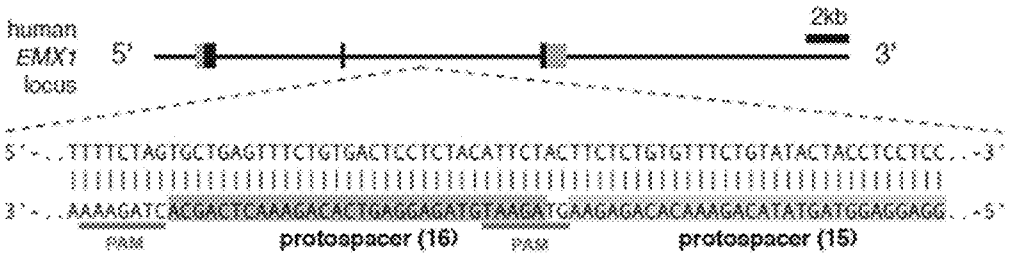

human
EMX1
locus
5'
2kb
3'
5'-..TTTTCTAGTGCTGAGTTTCTGTGACTCCTCTACATTCTACTTCTCTGTGTTTCTGTATACTACCTCCTCC..-3'
3'-..AAAAGATCACGACTCAAACACACTGAGCACATGTAACATGAAGACACACAAACACATATGATGGAGGAGG..-5'
PAM
protospacer (16)
PAM
protospacer (15)

FIG. 16

Primer name	Assay	Genomic Target	Primer sequence
Sp-EMX1-F	SURVEYOR assay, sequencing	*EMX1*	AAAACCACCCTTCTCTCTGGC
Sp-EMX1-R	SURVEYOR assay, sequencing	*EMX1*	GGAGATTGGAGACACGGAGAG
Sp-PVALB-F	SURVEYOR assay, sequencing	*PVALB*	CTGGAAAGCCAATGCCTGAC
Sp-PVALB-R	SURVEYOR assay, sequencing	*PVALB*	GGCAGCAAACTCCTTGTCCT
Sp-Tb-F	SURVEYOR assay, sequencing	*Tb*	GTGCTTTGCAGAGGCCTACC
Sp-Tb-R	SURVEYOR assay, sequencing	*Tb*	CCTGGAGCGCATGCAGTAGT
St-EMX1-F	SURVEYOR assay, sequencing	*EMX1*	ACCTTCTGTGTTTCCACCATTC
St-EMX1-R	SURVEYOR assay, sequencing	*EMX1*	TTGGGGAGTGCACAGACTTC
Sp-EMX1-RFLP-F	RFLP, sequencing	*EMX1*	GGCTCCCTGGGTTCAAAGTA
Sp-EMX1-RFLP-R	RFLP, sequencing	*EMX1*	AGAGGGGTCTGGATGTCGTAA
Pb_EMX1_sp1	Northern Blot Probe	Not applicable	TAGCTCTAAAACTTCTTCTTCTGCTCGGAC
Pb_tracrRNA	Northern Blot Probe	Not applicable	CTAGCCTTATTTTAACTTGCTATGCTGTTT

FIG. 17

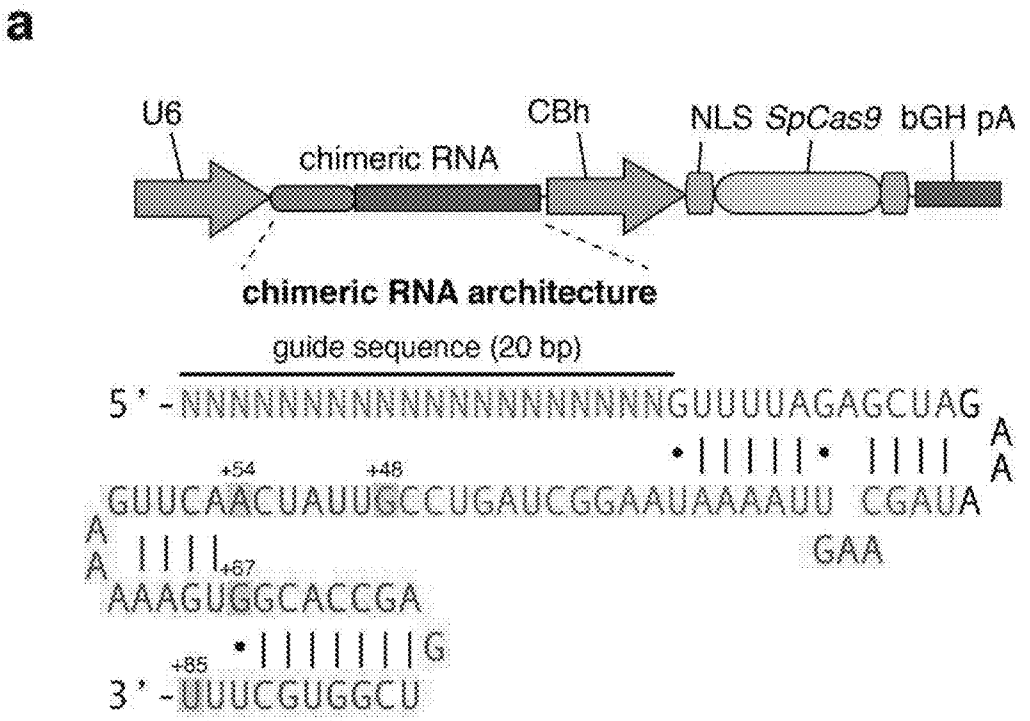

a
U6
CBh
NLS SpCas9 bGH pA
chimeric RNA
chimeric RNA architecture
guide sequence (20 bp)
5'-NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAG
A
A
+54
+48
GUUCAACUAUUGCCUGAUCGGAAUAAAAUU CGAUA
A
A
GAA
+67
AAAGUGGCACCGA
+85
G
3'-UUUCGUGGCU

FIG. 18A

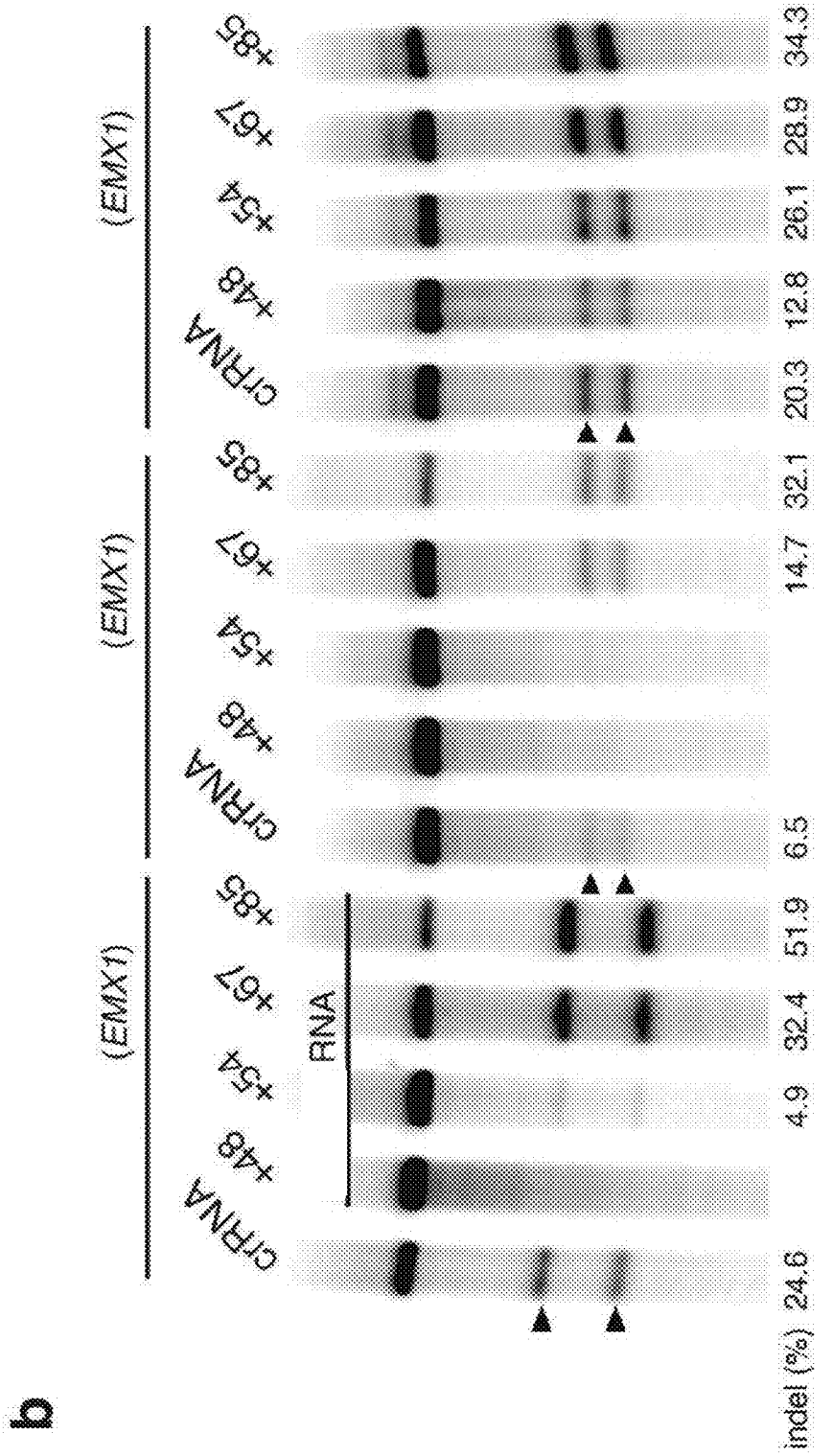

b
(EMX1)
(EMX1)
(EMX1)
crRNA
+48
+54
+67
+85
RNA
indel (%)
24.6
4.9
32.4
51.9
6.5
14.7
32.1
20.3
12.8
26.1
28.9
34.3

FIG. 18B

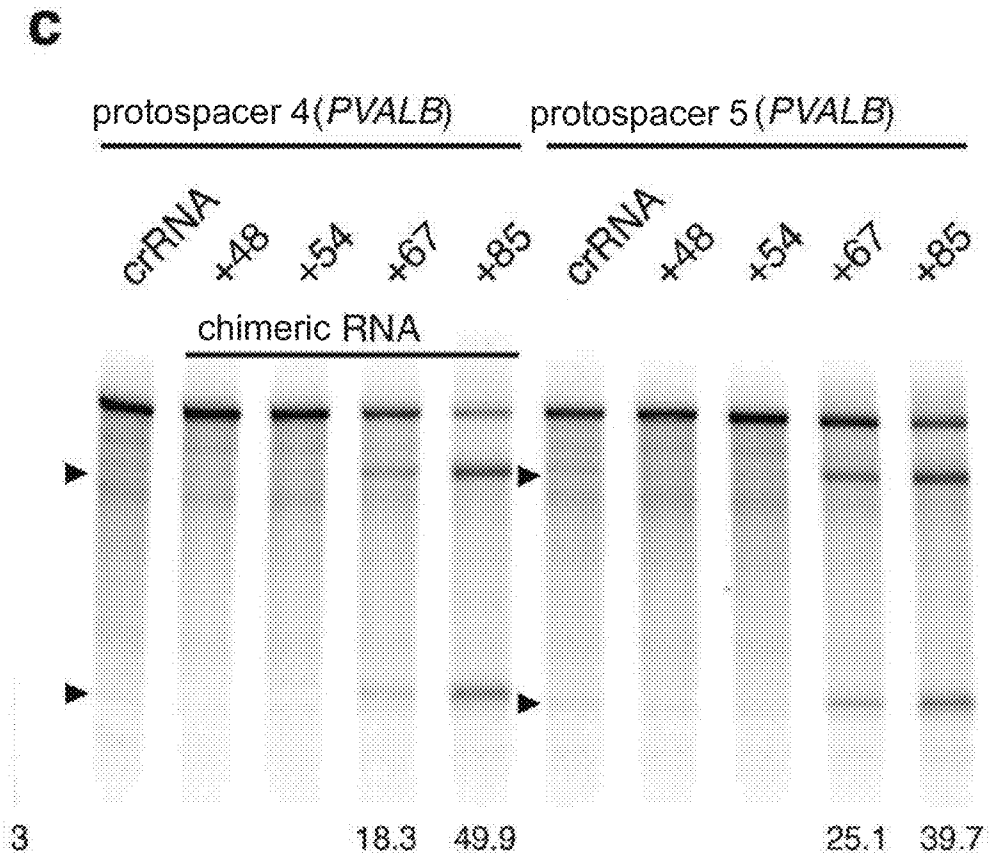

c
protospacer 4 (PVALB)
protospacer 5 (PVALB)
crRNA
+48
+54
+67
+85
crRNA
+48
+54
+67
+85
chimeric RNA
3
18.3
49.9
25.1
39.7

FIG. 18C

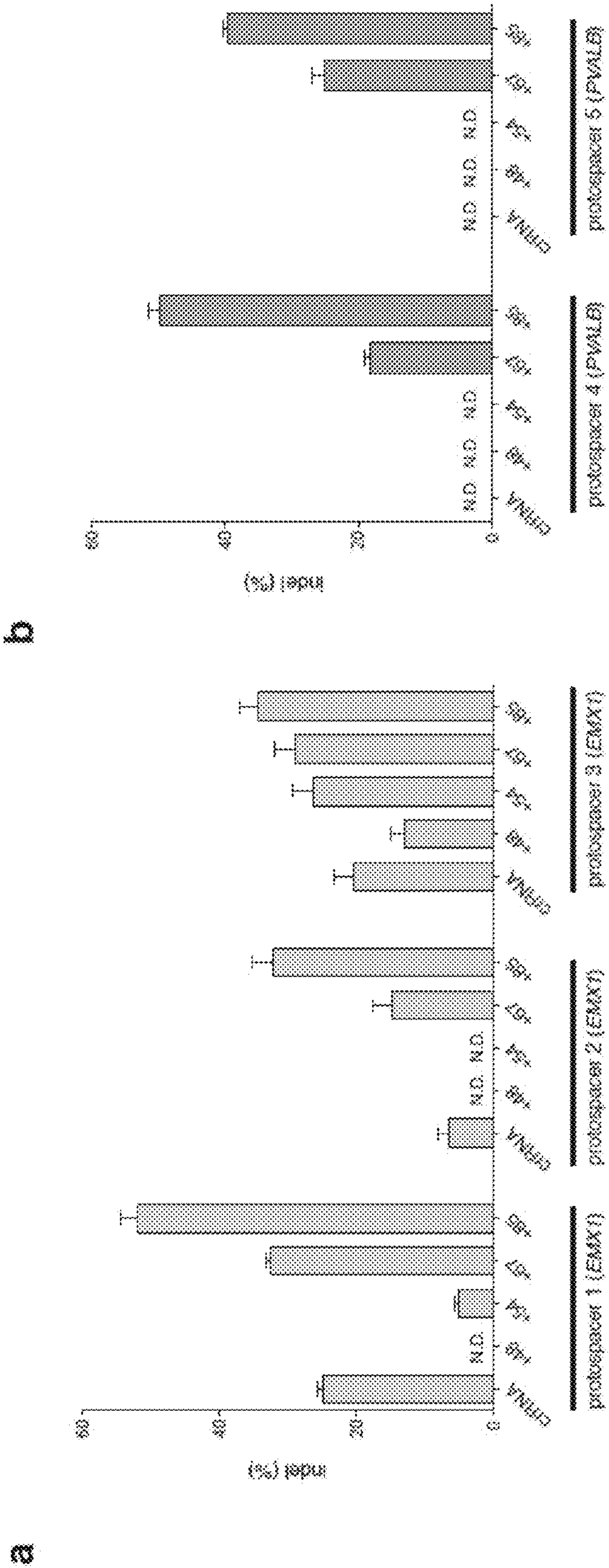
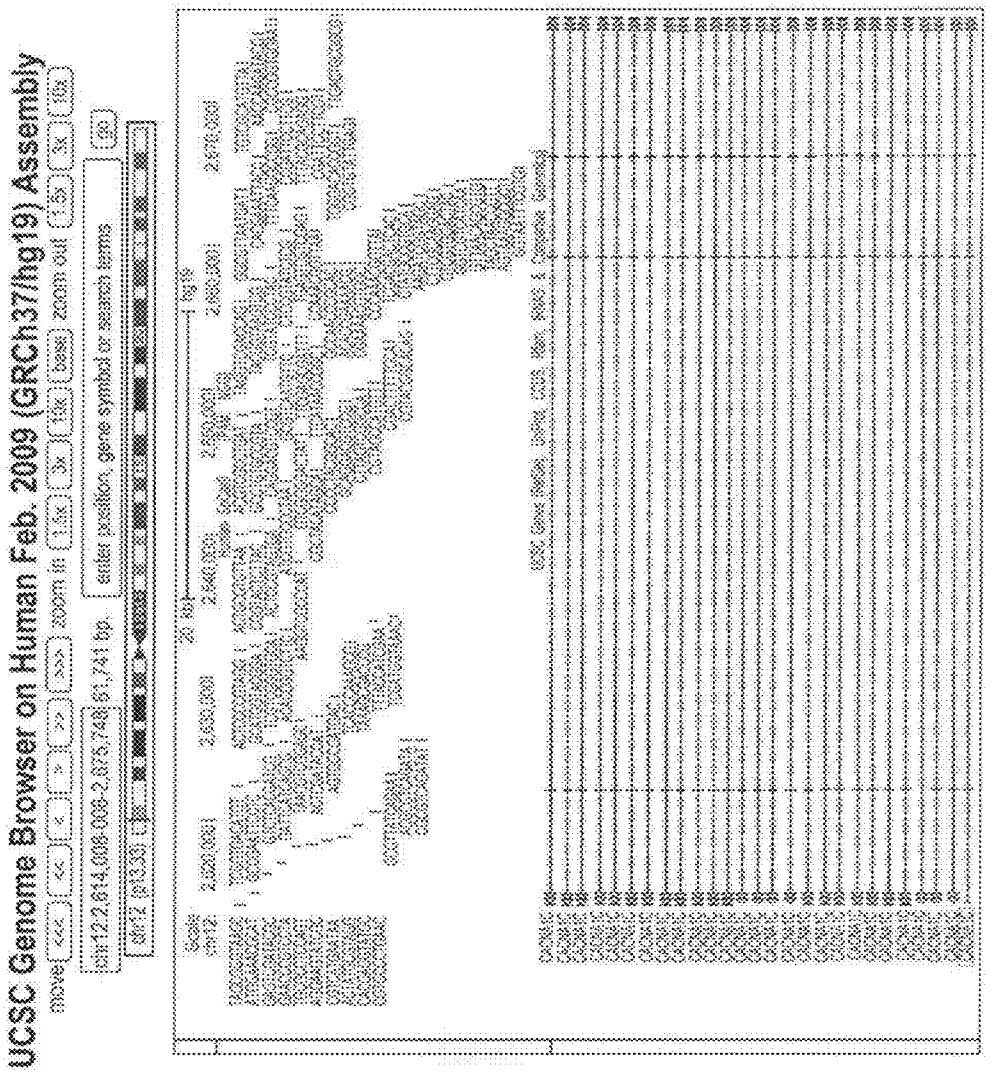

a
indel (%)
0
20
40
60
crRNA
N.D.
protospacer 1 (EMX1)
protospacer 2 (EMX1)
protospacer 3 (EMX1)
b
protospacer 4 (PVALB)
protospacer 5 (PVALB)

FIG. 19A-B

FIG. 20


UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly
move
zoom in
zoom out
enter position, gene symbol or search terms
go

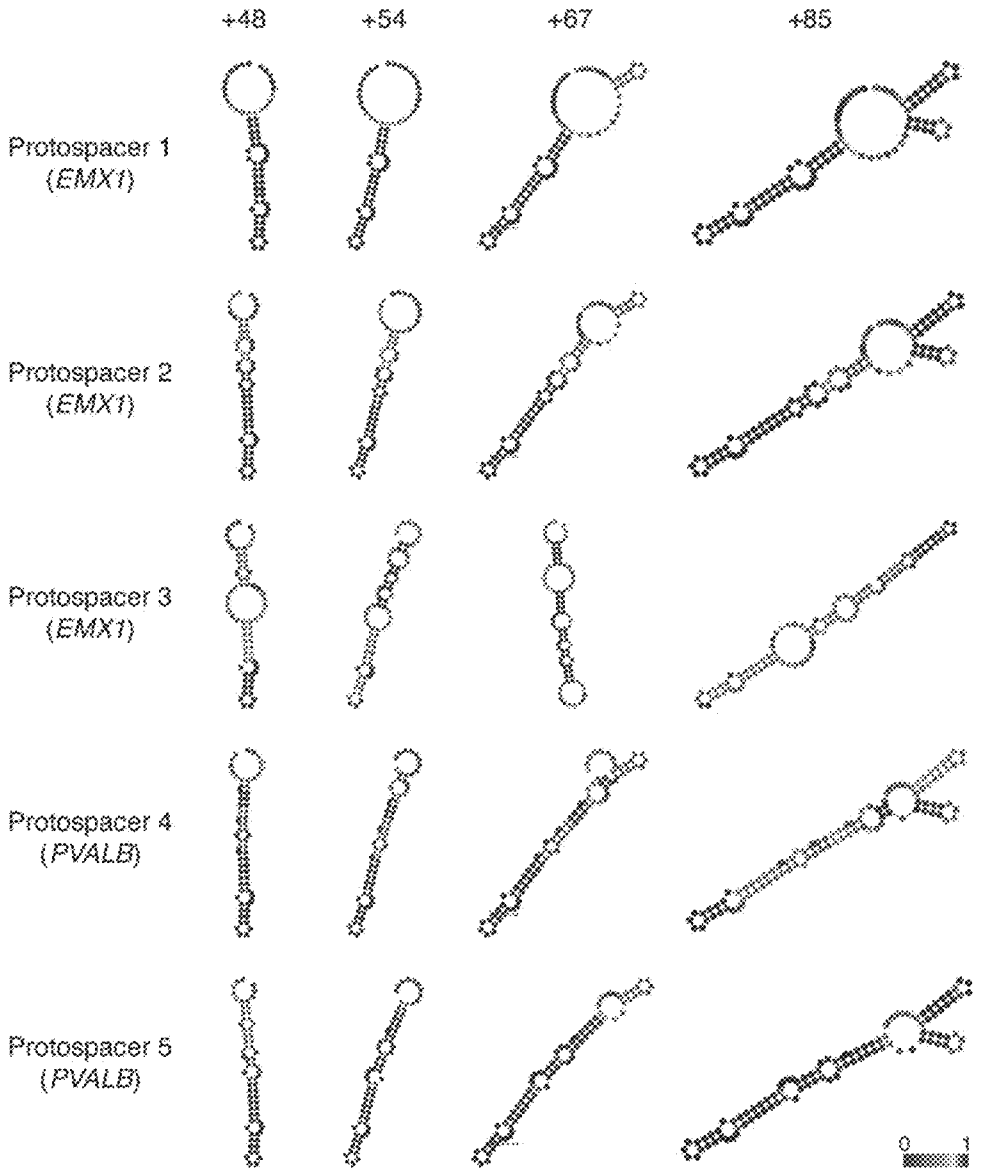

+48
+54
+67
+85
Protospacer 1 (EMX1)
Protospacer 2 (EMX1)
Protospacer 3 (EMX1)
Protospacer 4 (PVALB)
Protospacer 5 (PVALB)
0
1

FIG. 21

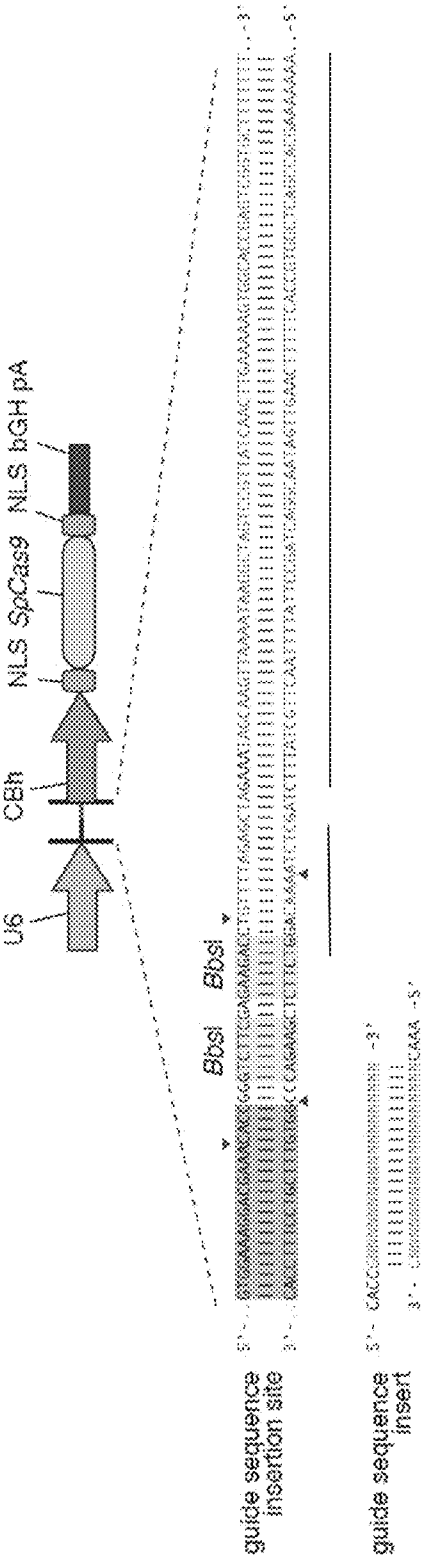
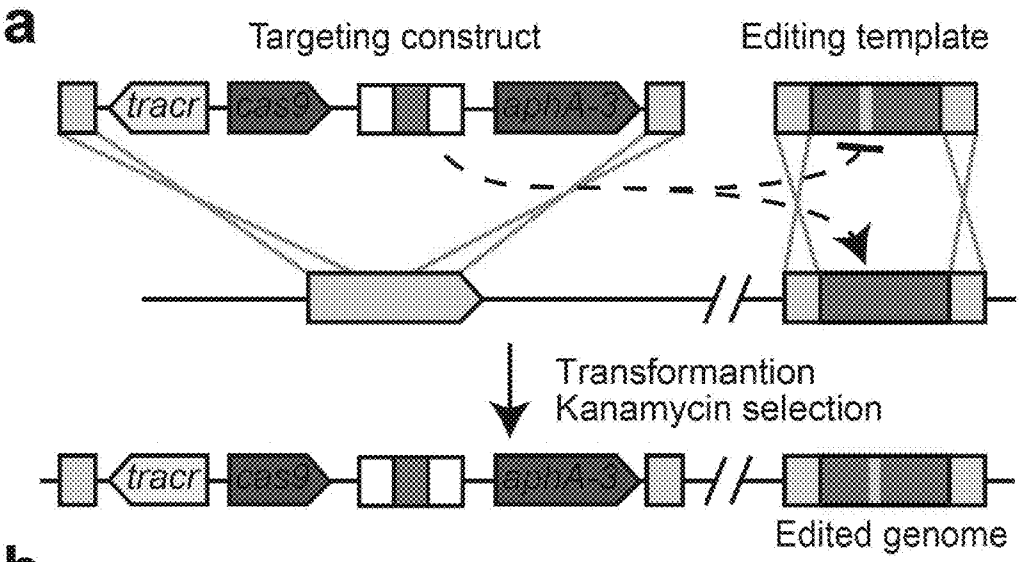

U6
CBh
NLS SpCas9
NLS bGH pA
BbsI
BbsI
guide sequence insertion site
guide sequence insert

FIG. 22



b

Editing Construct	cfu/ug of crR6M DNA
None	2.6 ± 0.9
R6(*srtA*)	14 ± 1.8
$R6^{370.1}$	25 ± 2.6

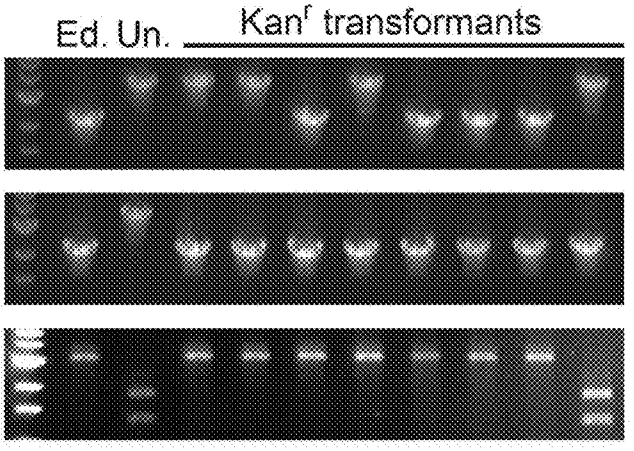
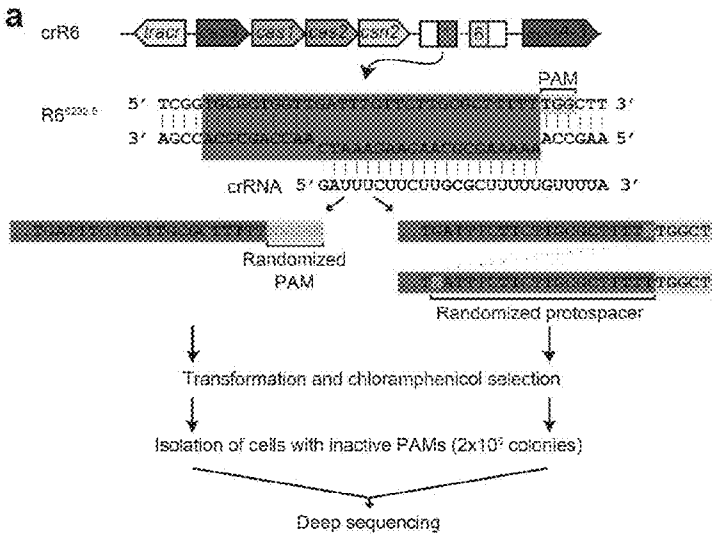


FIG. 23



b

2nd PAM position

1st PAM position	A		C		G		T		3rd PAM position
A	AAA	1.02	ACA	1.06	AGA	0.95	ATA	1.02	A
C	CAA	1.03	CCA	1.03	CGA	0.97	CTA	1.00	
G	GAA	1.10	GCA	1.09	GGA	0.93	GTA	1.01	
T	TAA	1.02	TCA	1.11	TGA	0.91	TTA	1.11	
A	AAC	1.07	ACC	1.02	AGC	1.00	ATC	1.09	C
C	CAC	1.06	CCC	1.04	CGC	1.00	CTC	1.09	
G	GAC	1.05	GCC	1.02	GGC	1.02	GTC	1.22	
T	TAC	1.06	TCC	1.05	TGC	1.03	TTC	1.06	
A	AAG	0.48	ACG	0.97	AGG	0.08	ATG	0.96	G
C	CAG	0.78	CCG	1.08	CGG	0.06	CTG	1.03	
G	GAG	0.80	GCG	1.00	GGG	0.06	GTG	1.20	
T	TAG	0.86	TCG	0.91	TGG	0.07	TTG	1.06	
A	AAT	1.01	ACT	1.09	AGT	1.04	ATT	1.03	T
C	CAT	1.03	CCT	1.03	CGT	1.07	CTT	1.08	
G	GAT	1.04	GCT	1.08	GGT	1.08	GTT	1.12	
T	TAT	1.06	TCT	1.03	TGT	1.06	TTT	1.01	

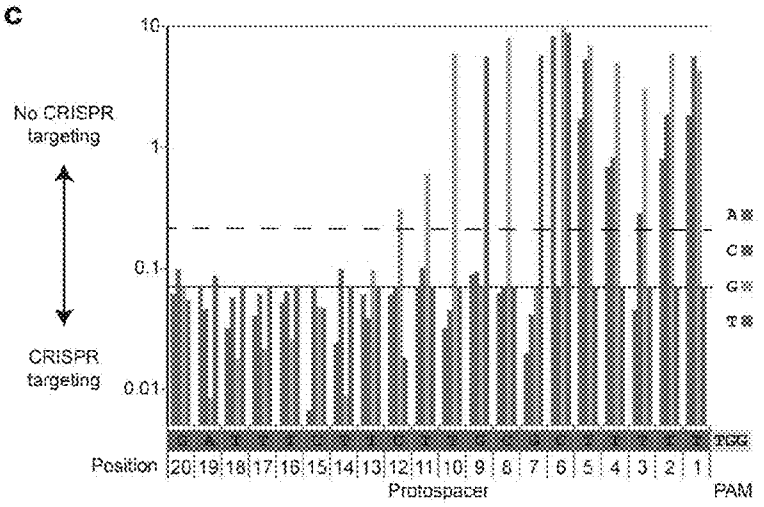


FIG. 24

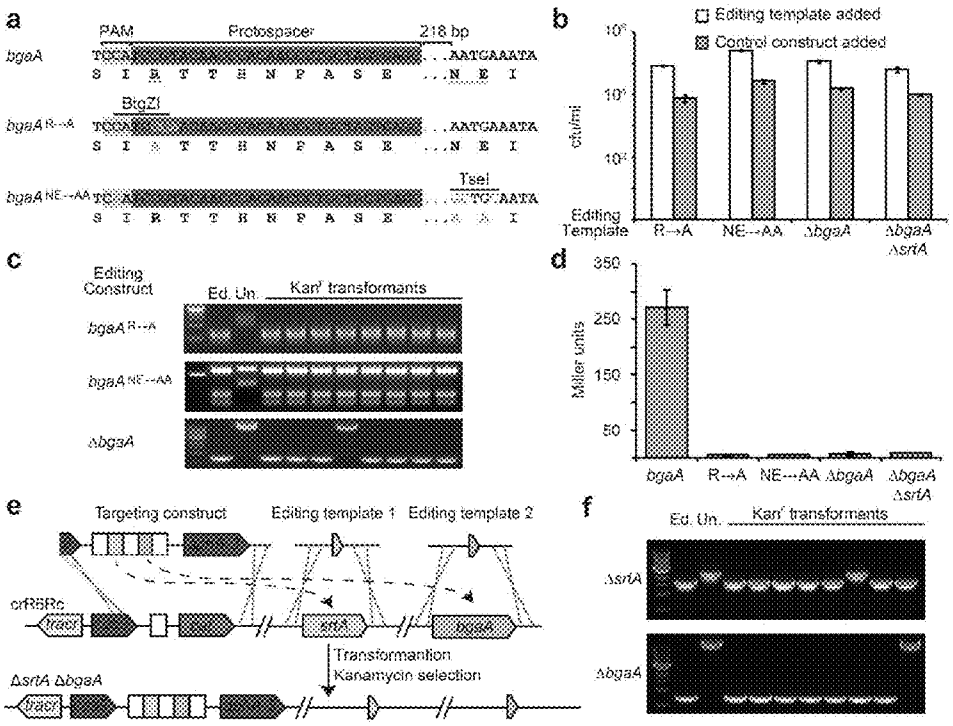

a
PAM
Protospacer
218 bp
bgaA
TCCA
AATGAAATA
S I R T T H N P A S E
N E I
BtgZI
bgaA R→A
TCCA
AATGAAATA
S I S T T H N P A S E
N E I
TseI
bgaA NE→AA
TCCA
TGCAATA
S I R T T H N P A S E
A A I
b
Editing template added
Control construct added
10⁵
10⁴
10³
cfu/ml
Editing Template
R→A
NE→AA
ΔbgaA
ΔbgaA ΔsrtA
c
Editing Construct
Ed. Un.
Kan^r transformants
bgaA R→A
bgaA NE→AA
ΔbgaA
d
350
250
150
50
Miller units
bgaA
R→A
NE→AA
ΔbgaA
ΔbgaA ΔsrtA
e
Targeting construct
Editing template 1
Editing template 2
crRBRc
tracrI
srtA
bgaA
Transformation
Kanamycin selection
ΔsrtA ΔbgaA
tracrI
f
Ed. Un.
Kan^r transformants
ΔsrtA
ΔbgaA

FIG. 25

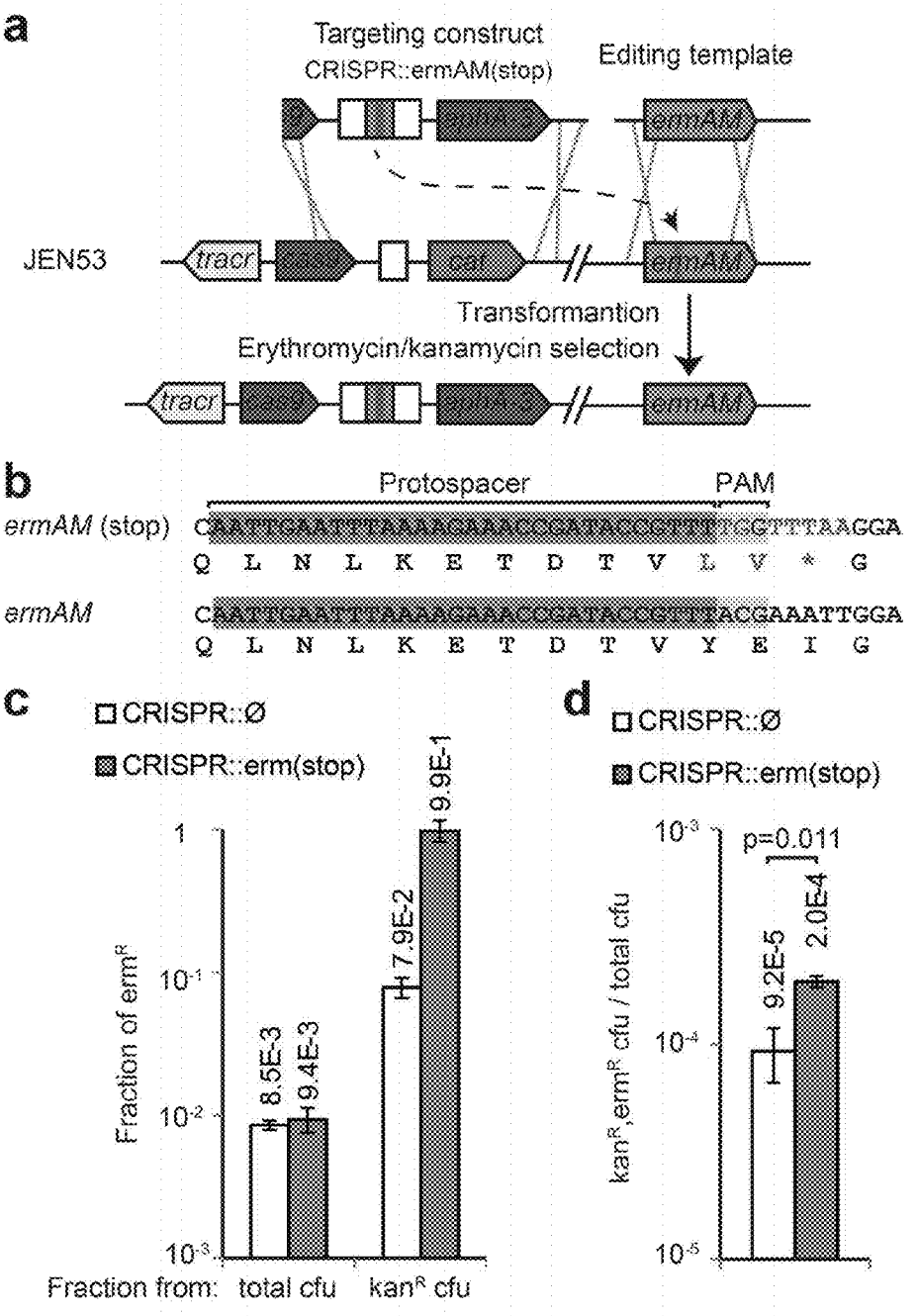

a
Targeting construct
CRISPR::ermAM(stop)
Editing template
ermAM
JEN53
tracr
ermAM
Transformantion
Erythromycin/kanamycin selection
tracr
ermAM
b
Protospacer
PAM
ermAM (stop)
Q L N L K E T D T V L V * G
ermAM
Q L N L K E T D T V Y E I G
c
CRISPR::Ø
CRISPR::erm(stop)
Fraction of ermR
1
10-1
10-2
10-3
8.5E-3
9.4E-3
7.9E-2
9.9E-1
Fraction from: total cfu kanR cfu
d
CRISPR::Ø
CRISPR::erm(stop)
kanR,ermR cfu / total cfu
10-3
10-4
10-5
p=0.011
9.2E-5
2.0E-4

FIG. 26

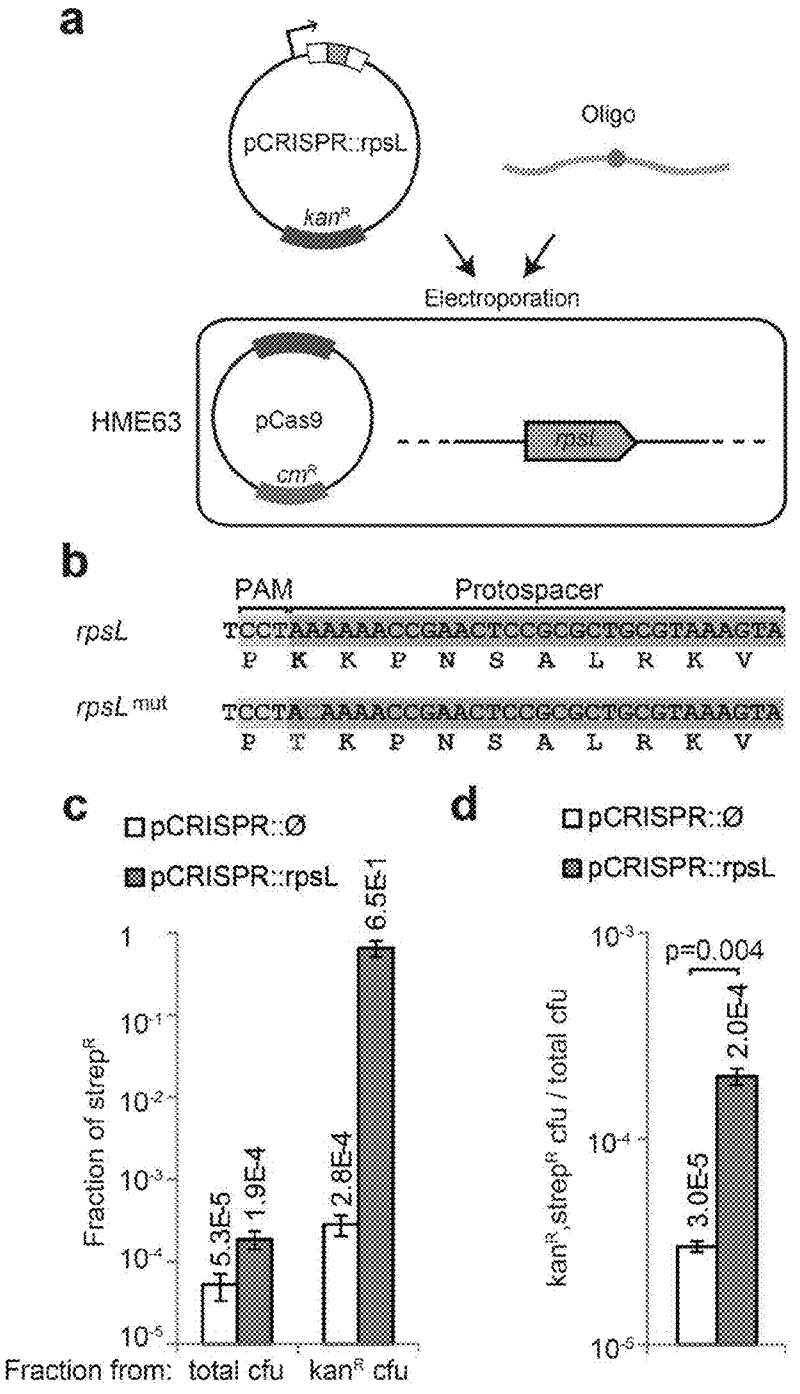

a
pCRISPR::rpsL
kanR
Oligo
Electroporation
HME63
pCas9
cmR
rpsL
b
PAM
Protospacer
rpsL
TCCTAAAAAACCGAACTCCGCGCTGCGTAAAGTA
P K K P N S A L R K V
rpsL mut
TCCTA AAAACCGAACTCCGCGCTGCGTAAAGTA
P T K P N S A L R K V
c
pCRISPR::Ø
pCRISPR::rpsL
Fraction of strepR
1
10-1
10-2
10-3
10-4
10-5
5.3E-5
1.9E-4
2.8E-4
6.5E-1
Fraction from: total cfu
kanR cfu
d
pCRISPR::Ø
pCRISPR::rpsL
kanR,strepR cfu / total cfu
10-3
10-4
10-5
p=0.004
3.0E-5
2.0E-4

FIG. 27

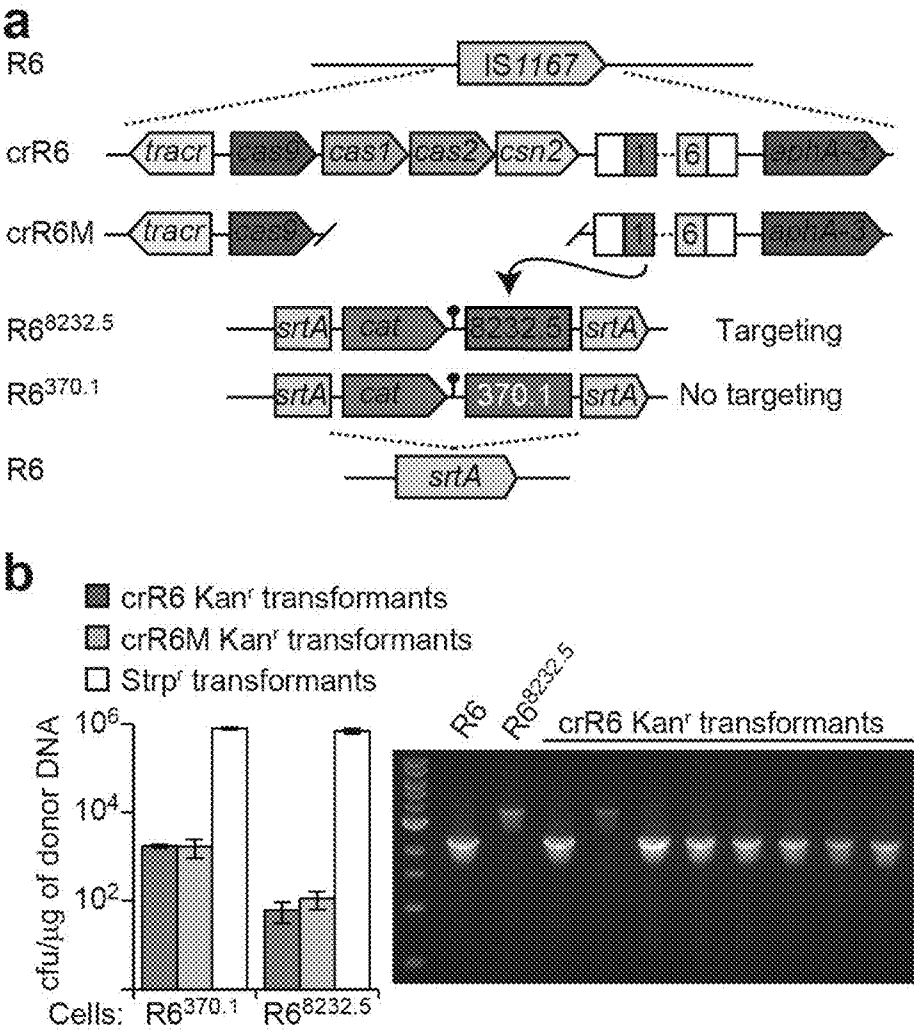

a
R6
IS1167
crR6
tracr
cas1
cas2
csn2
6
crR6M
tracr
6
R6^8232.5
srtA
cat
srtA
Targeting
R6^370.1
srtA
cat
370.1
srtA
No targeting
R6
srtA
b
crR6 Kan^r transformants
crR6M Kan^r transformants
Strp^r transformants
cfu/μg of donor DNA
10^6
10^4
10^2
Cells: R6^370.1
R6^8232.5
R6
R6^8232.5
crR6 Kan^r transformants

FIG. 28

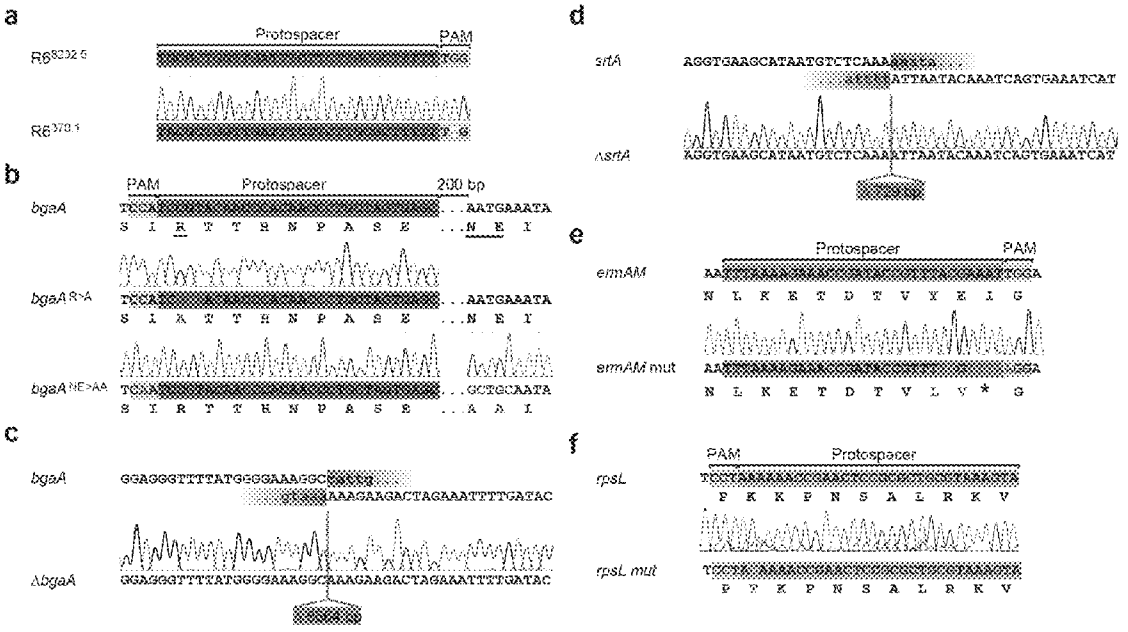

a
Protospacer
PAM
b
PAM
Protospacer
200 bp
bgaA
bgaA R>A
bgaA NE>AA
c
bgaA
ΔbgaA
d
srtA
ΔsrtA
e
ermAM
ermAM mut
f
PAM
Protospacer
rpsL
rpsL mut

FIG. 29

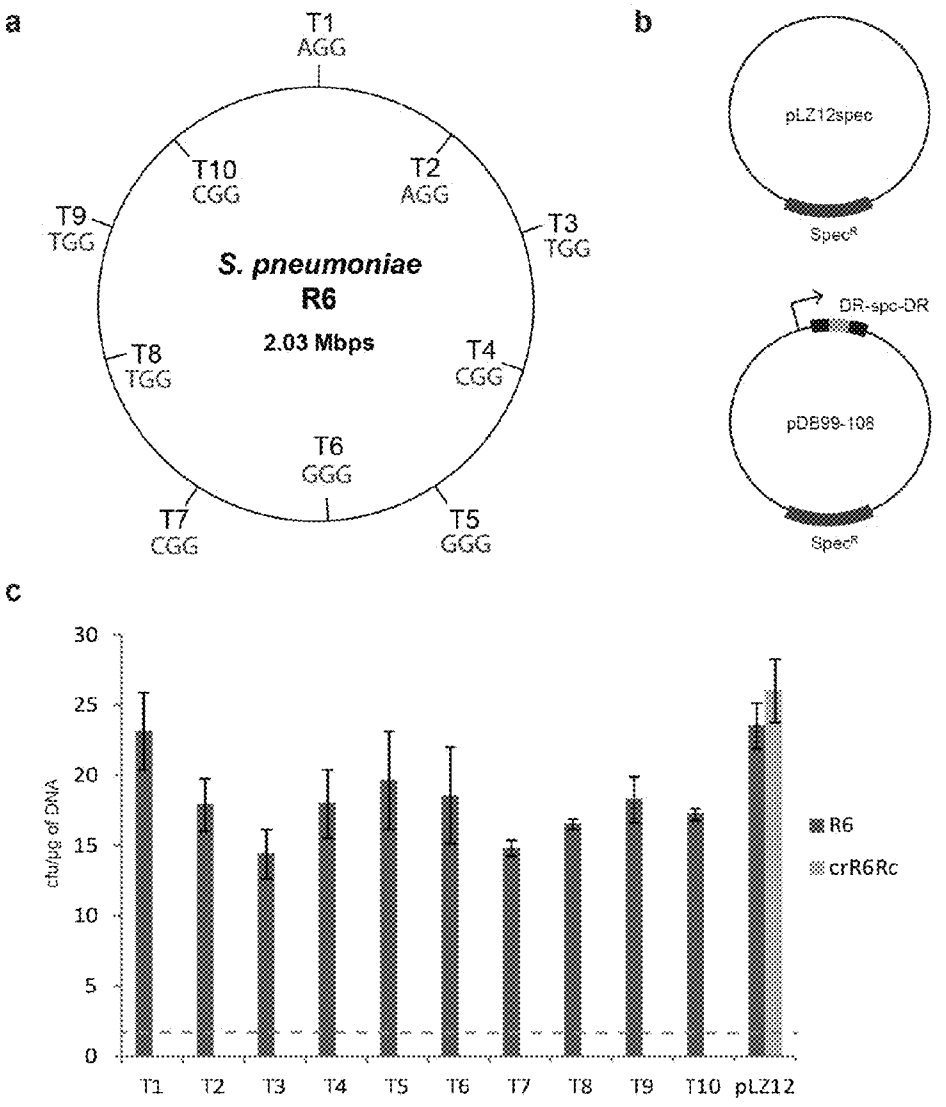

a
T1
AGG
T2
AGG
T3
TGG
T4
CGG
T5
GGG
T6
GGG
T7
CGG
T8
TGG
T9
TGG
T10
CGG
S. pneumoniae
R6
2.03 Mbps
b
pLZ12spec
Spec^R
DR-spc-DR
pDB99-108
Spec^R
c
cfu/µg of DNA
30
25
20
15
10
5
0
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
pLZ12
R6
crR6Rc

FIG. 30

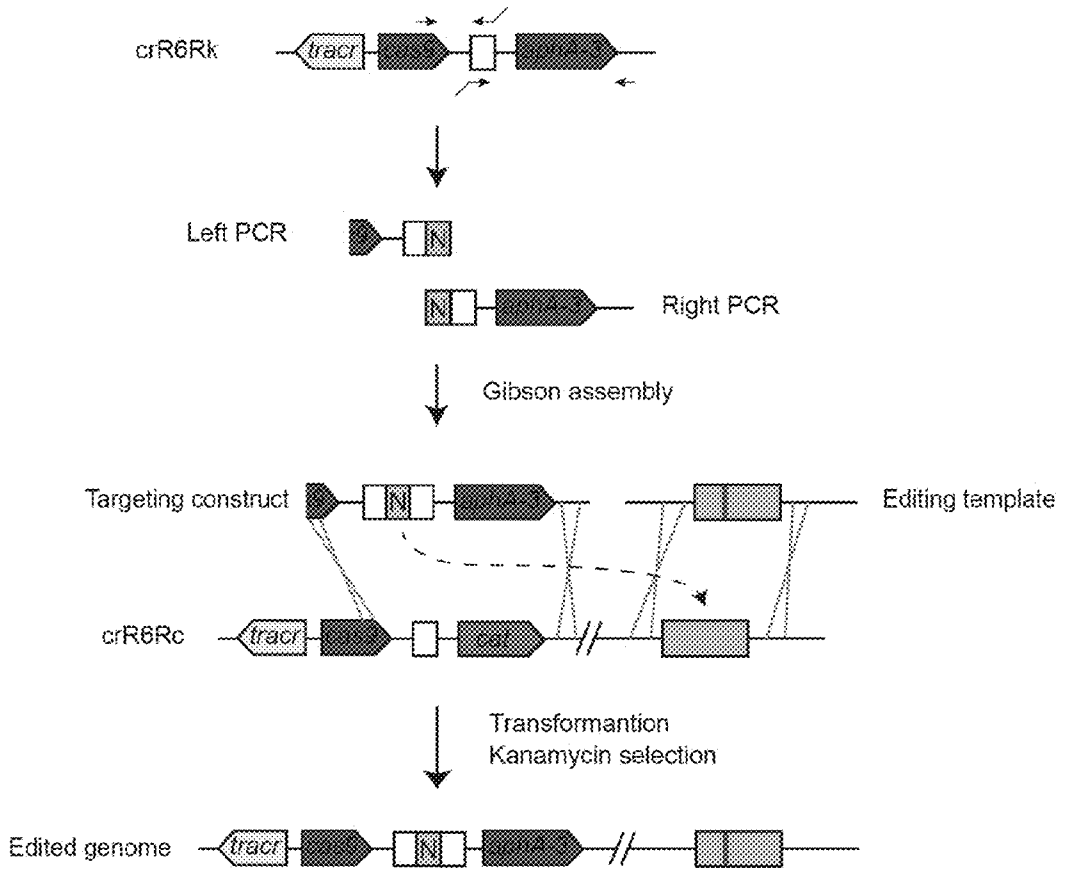

crR6Rk
tracr
Left PCR
N
Right PCR
Gibson assembly
Targeting construct
Editing template
crR6Rc
Transformantion
Kanamycin selection
Edited genome

FIG. 31

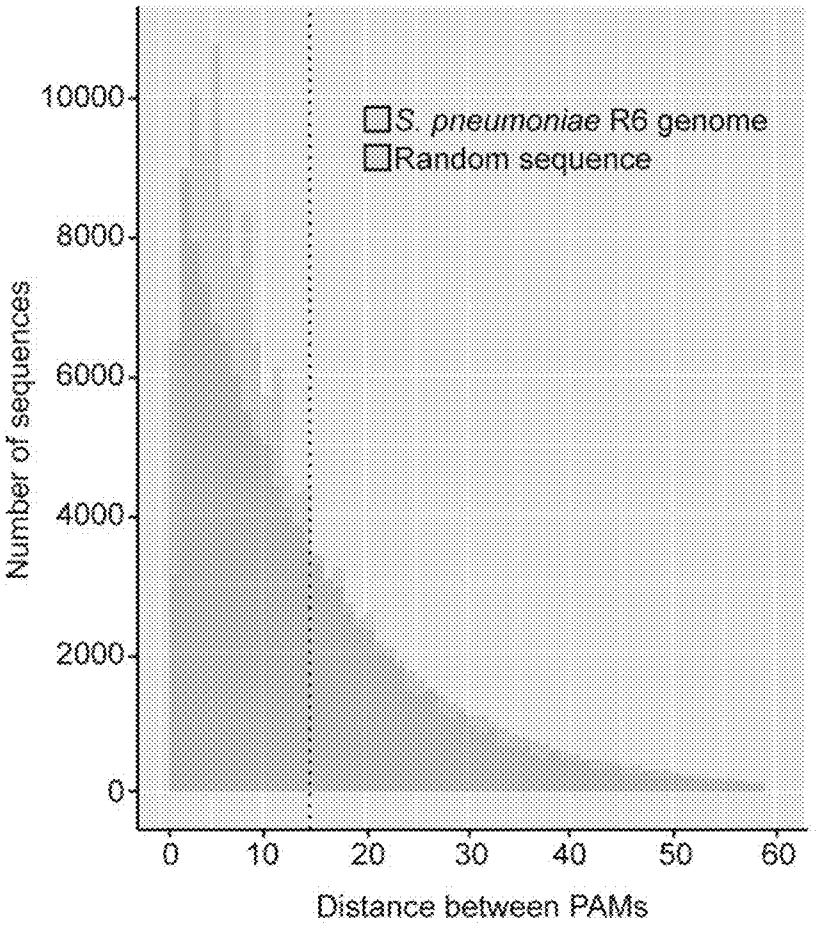

10000
8000
6000
4000
2000
0
Number of sequences
S. pneumoniae R6 genome
Random sequence
0
10
20
30
40
50
60
Distance between PAMs

FIG. 32

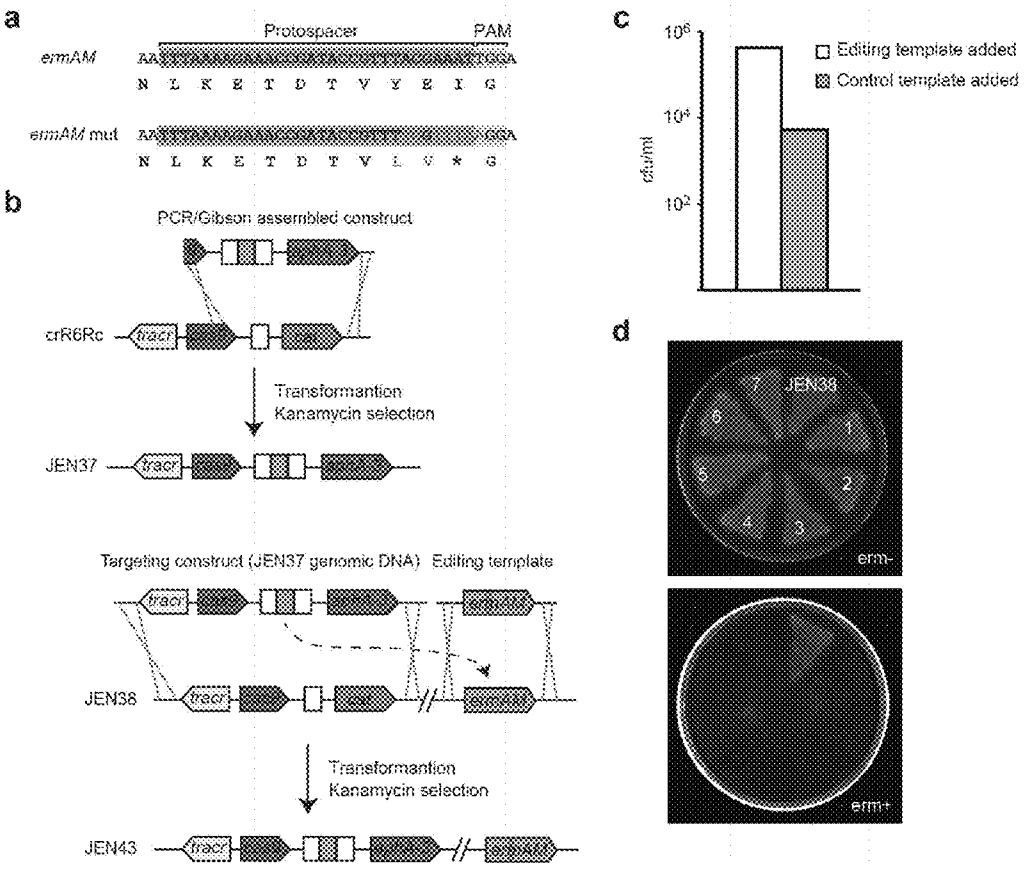

a
Protospacer
PAM
ermAM
N L K E T D T V Y E I G
ermAM mut
N L K E T D T V L V * G
b
PCR/Gibson assembled construct
crR6Rc
tracr
Transformantion
Kanamycin selection
JEN37
Targeting construct (JEN37 genomic DNA) Editing template
JEN38
Transformantion
Kanamycin selection
JEN43
c
cfu/ml
Editing template added
Control template added
d
JEN38
erm-
erm+

FIG. 33

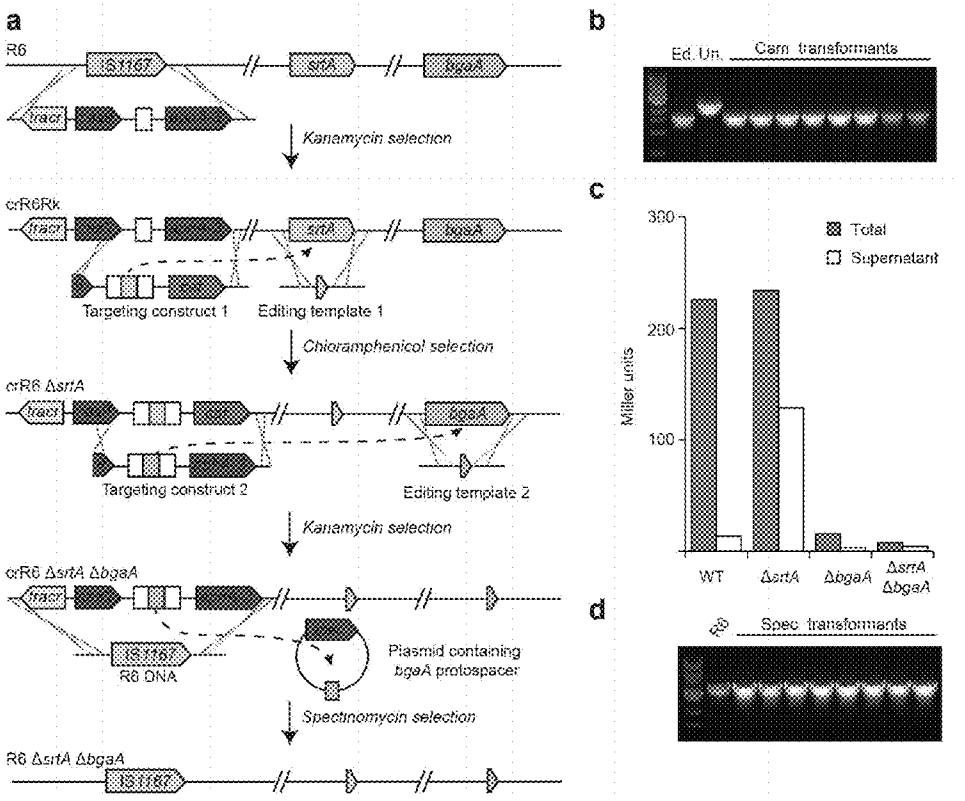

a
R6
IS1167
srtA
bgaA
tracr
Kanamycin selection
crR6Rk
Targeting construct 1
Editing template 1
Chloramphenicol selection
crR6 ΔsrtA
Targeting construct 2
Editing template 2
Kanamycin selection
crR6 ΔsrtA ΔbgaA
R6 DNA
Plasmid containing
bgaA protospacer
Spectinomycin selection
R6 ΔsrtA ΔbgaA
b
Ed. Un.
Cam transformants
c
Total
Supernatant
Miller units
300
200
100
WT
ΔsrtA
ΔbgaA
ΔsrtA
ΔbgaA
d
Spec transformants

FIG. 34

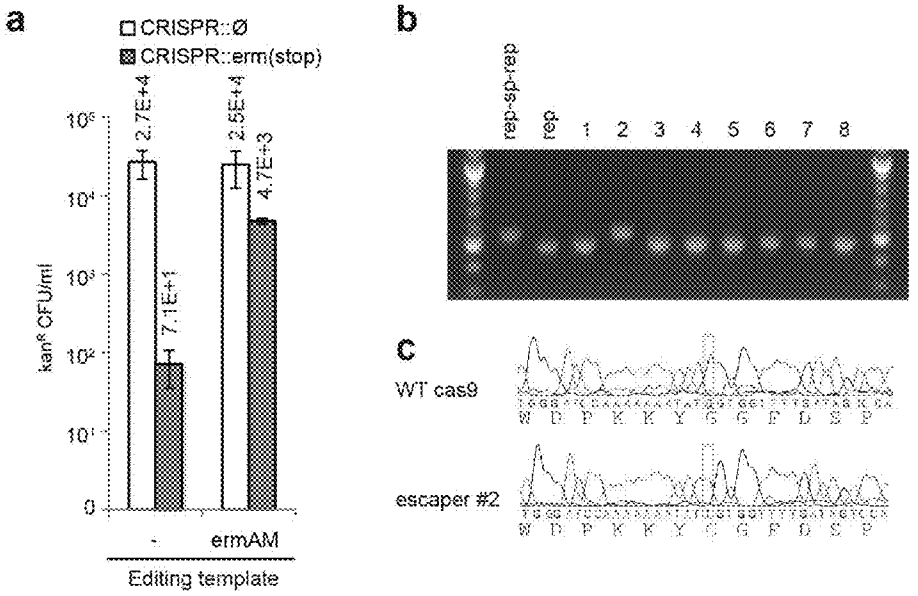

a
CRISPR::Ø
CRISPR::erm(stop)
2.7E+4
7.1E+1
2.5E+4
4.7E+3
kan^R CFU/ml
-
ermAM
Editing template
b
rep-sp-rep
rep
1 2 3 4 5 6 7 8
c
WT cas9
escaper #2

FIG. 35

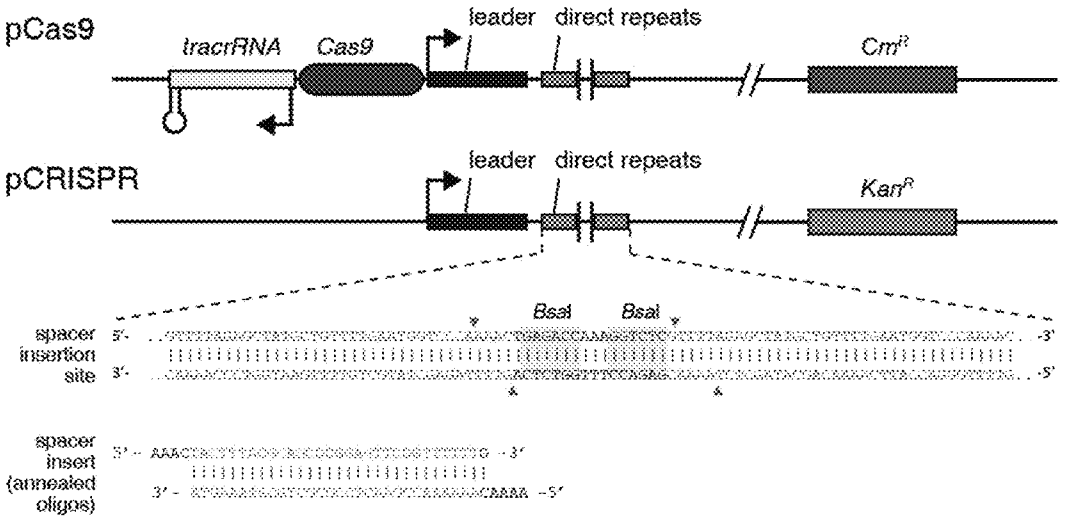

pCas9
tracrRNA
Cas9
leader
direct repeats
Cm^R
pCRISPR
leader
direct repeats
Kan^R
BsaI
BsaI
spacer insertion site
5'-
3'-
-3'
-5'
spacer insert (annealed oligos)
5' -
3' -
-3'
-5'

FIG. 36

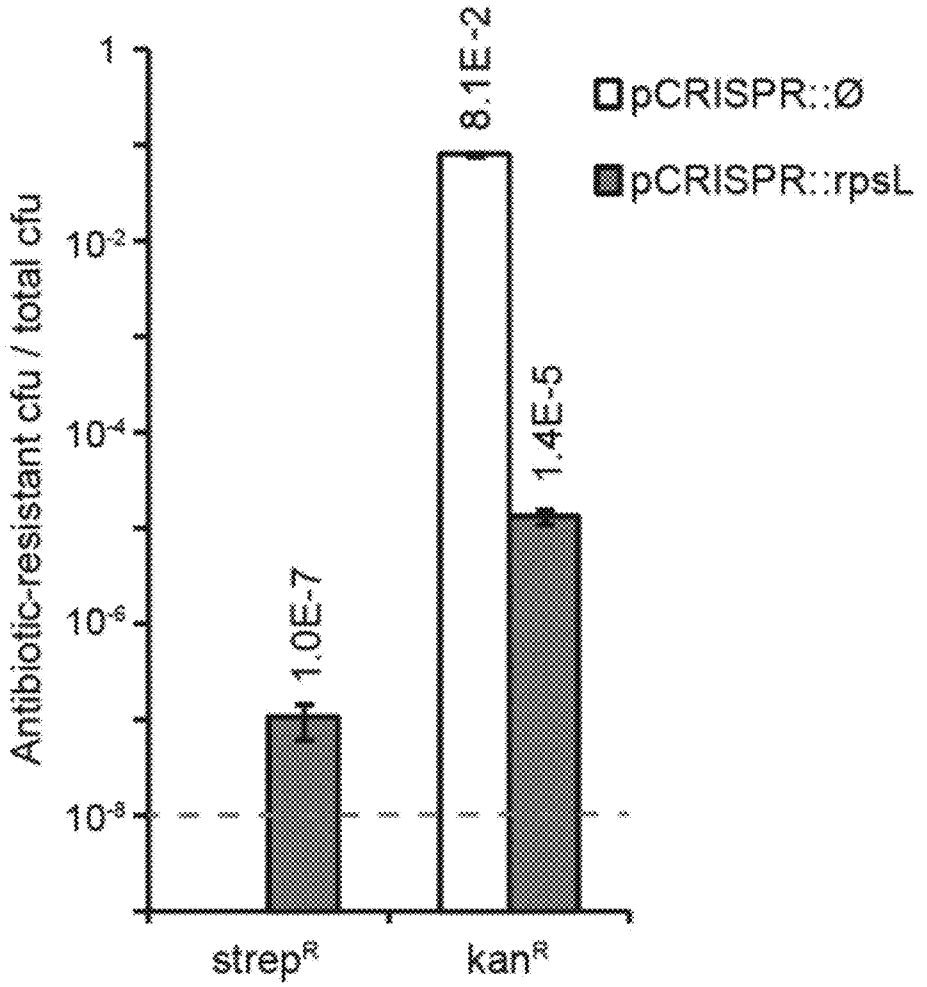

Antibiotic-resistant cfu / total cfu
1
10-2
10-4
10-6
10-8
8.1E-2
1.4E-5
1.0E-7
pCRISPR::Ø
pCRISPR::rpsL
strepR
kanR

FIG. 37

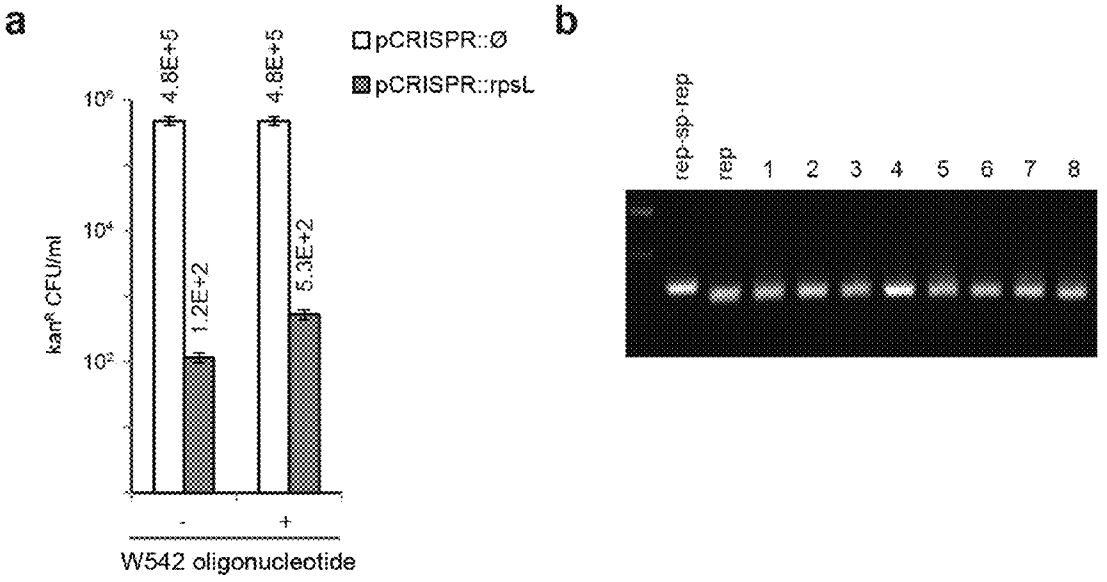

a
pCRISPR::Ø
pCRISPR::rpsL
4.8E+5
4.8E+5
1.2E+2
5.3E+2
kan^R CFU/ml
W542 oligonucleotide
b
rep-sp-rep
rep
1
2
3
4
5
6
7
8

FIG. 38

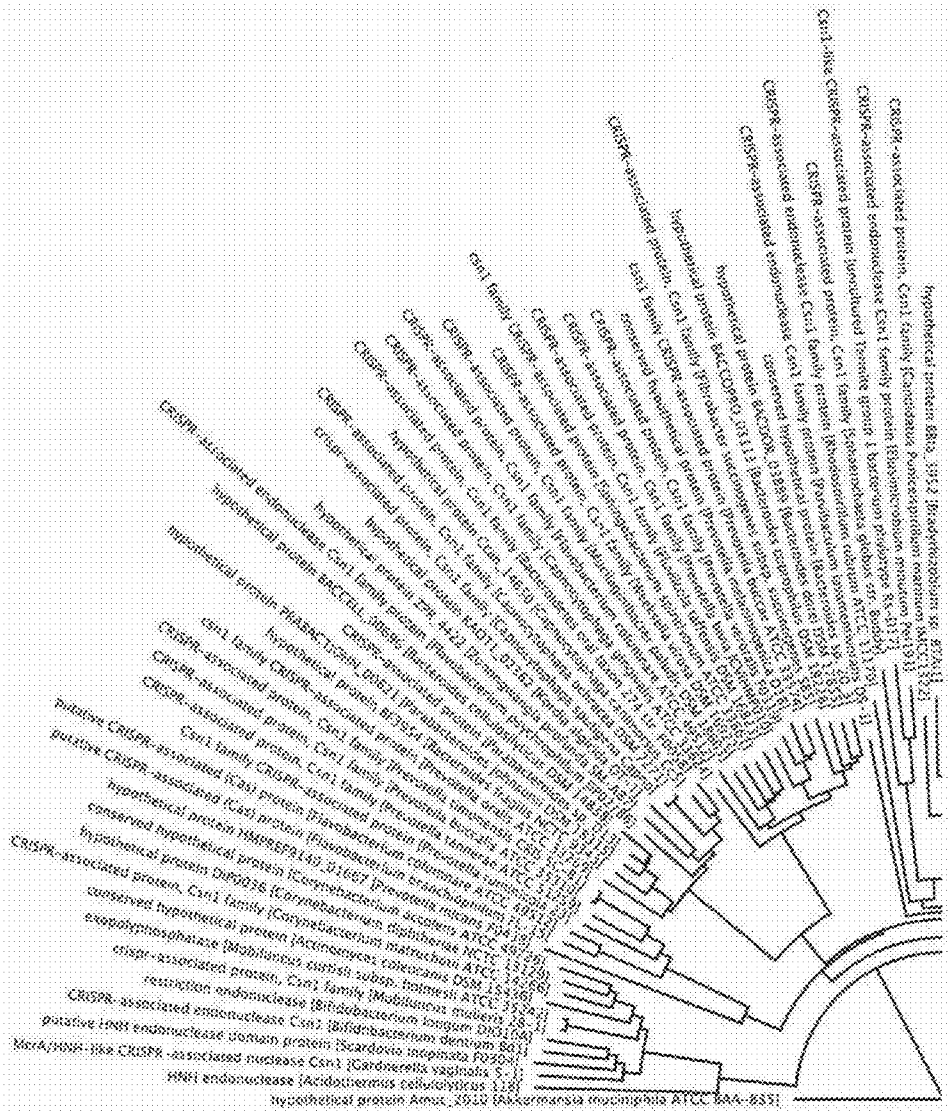


FIG. 39A

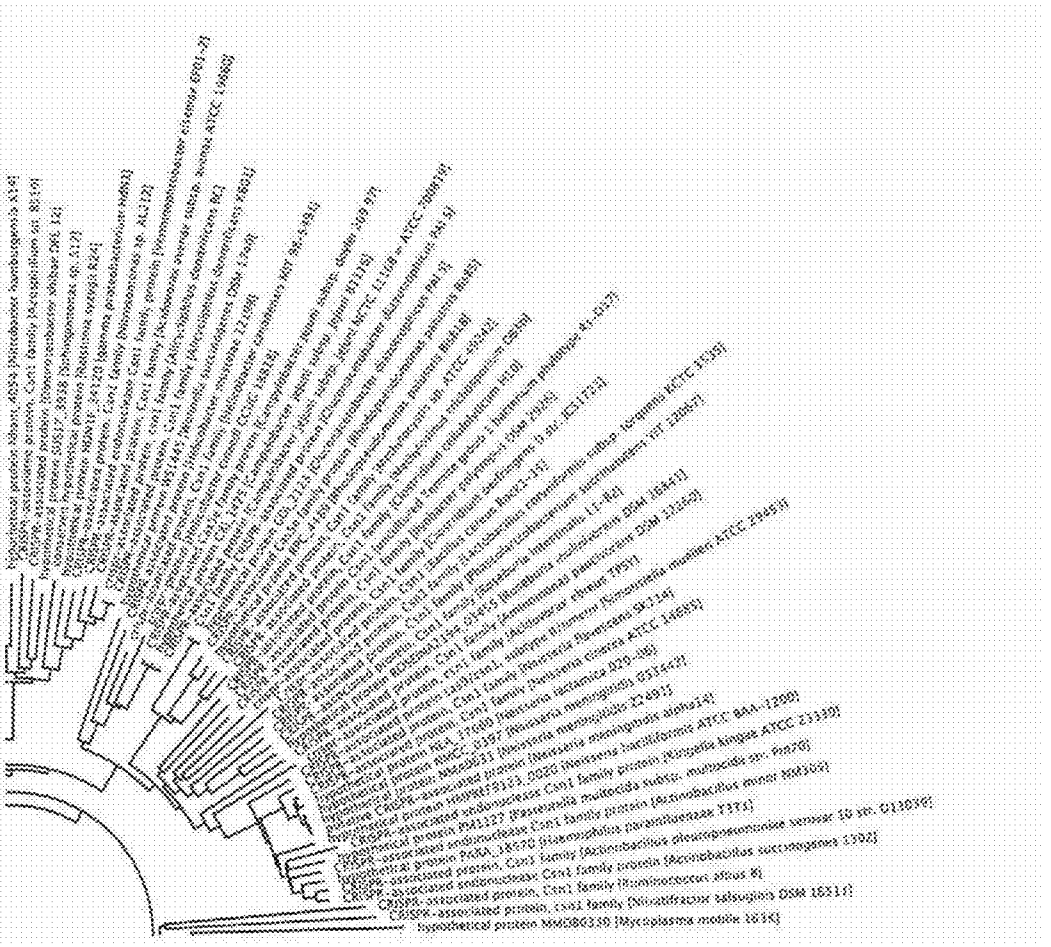


FIG. 39B

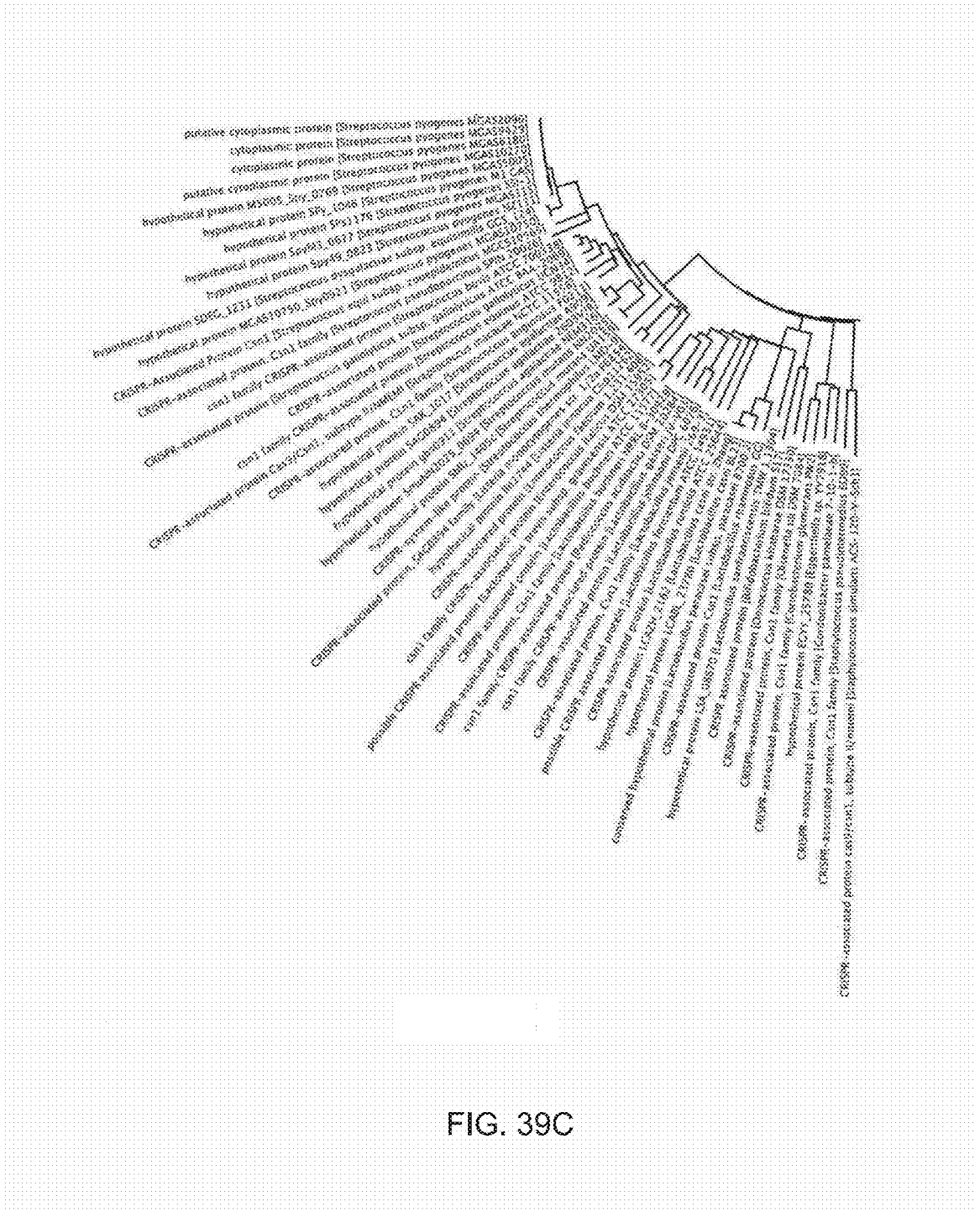


FIG. 39C

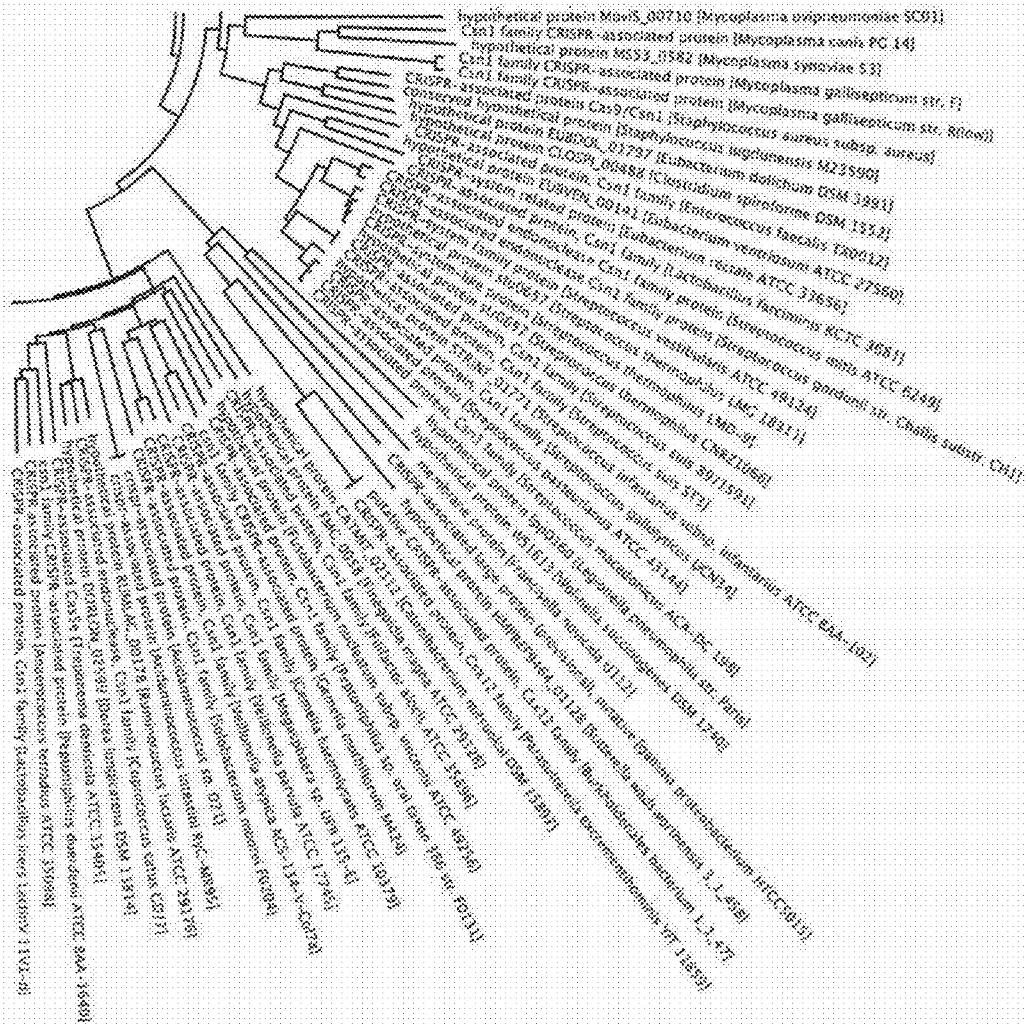


FIG. 39D

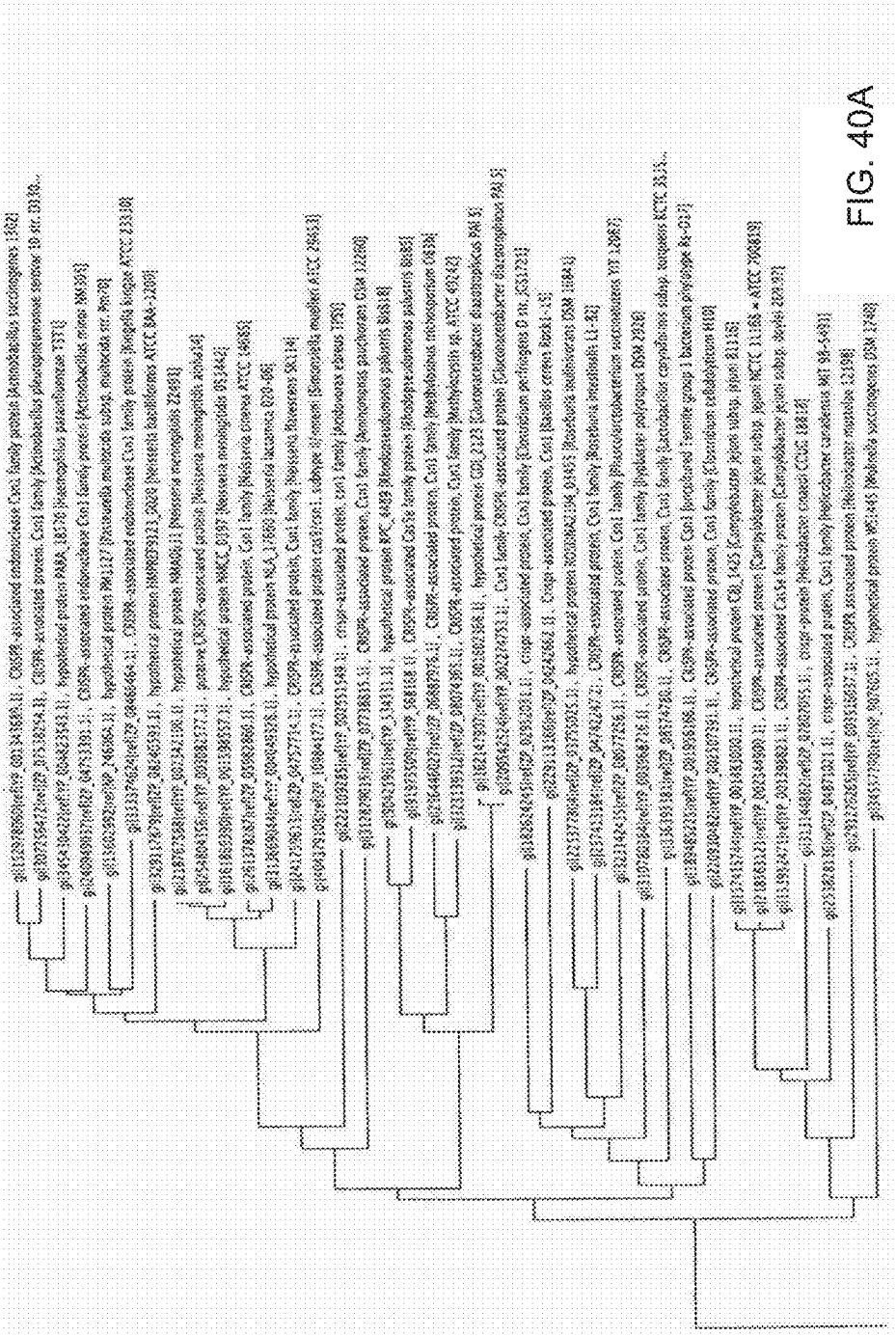


FIG. 40A

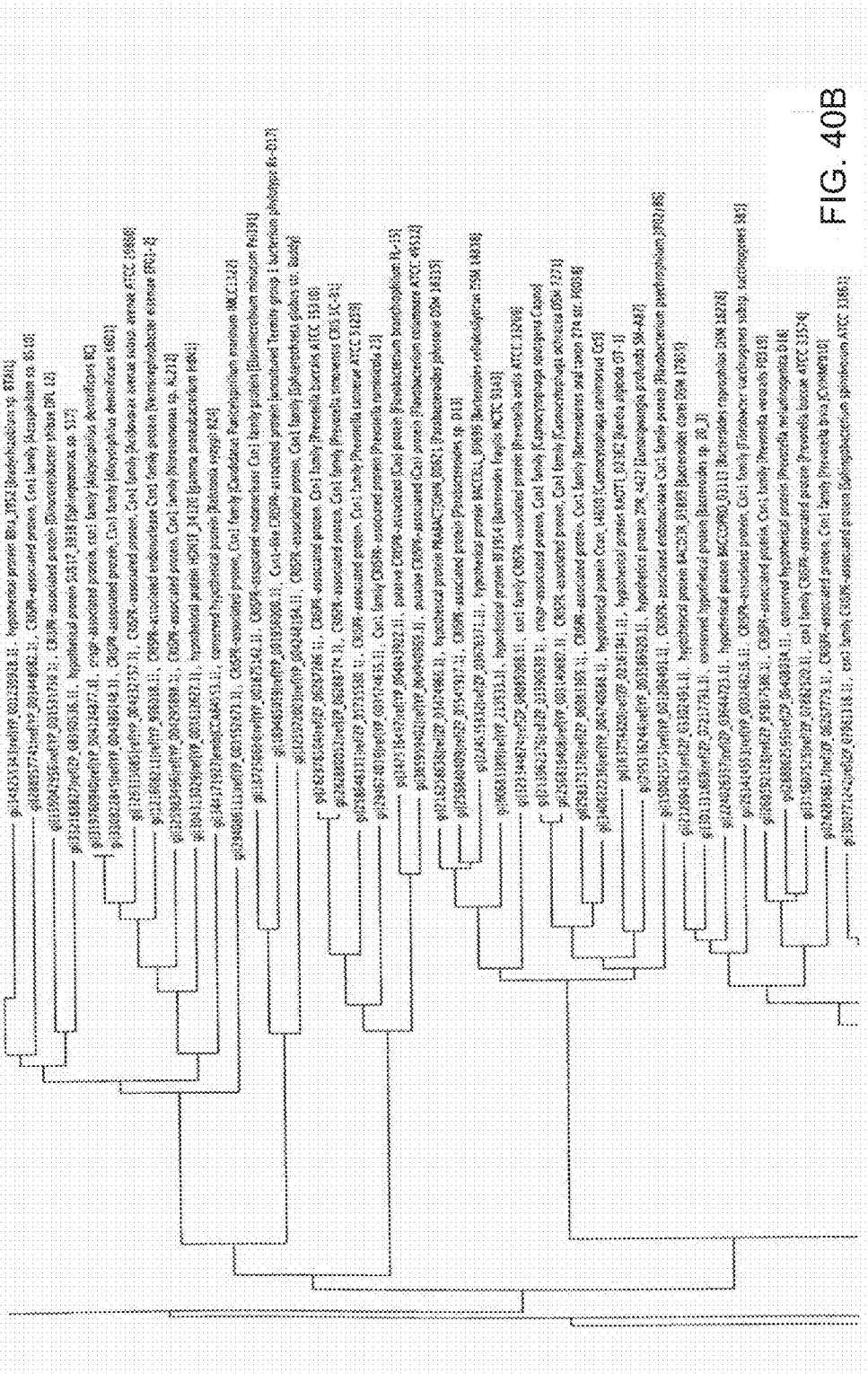


FIG. 40B

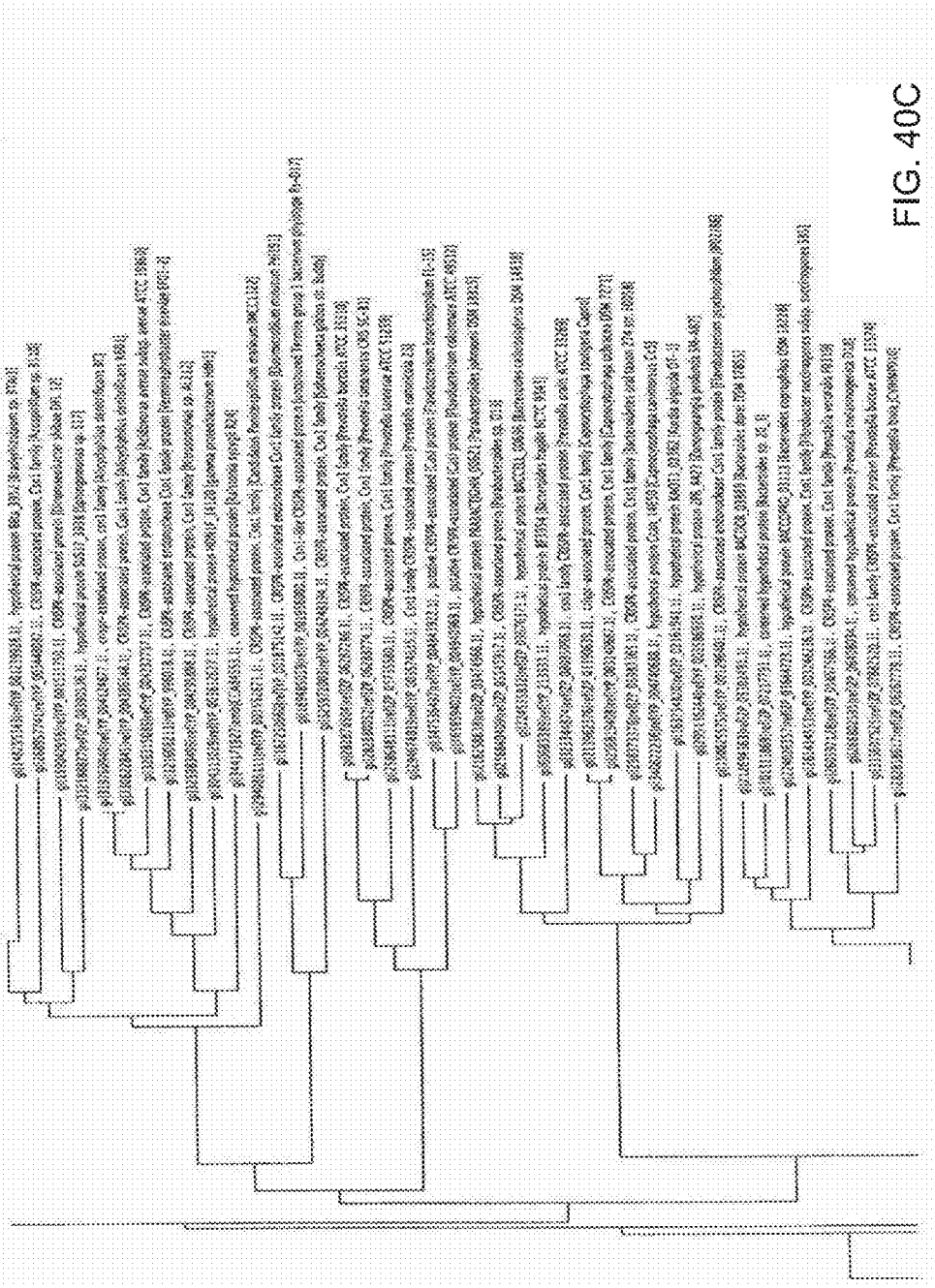


FIG. 40C

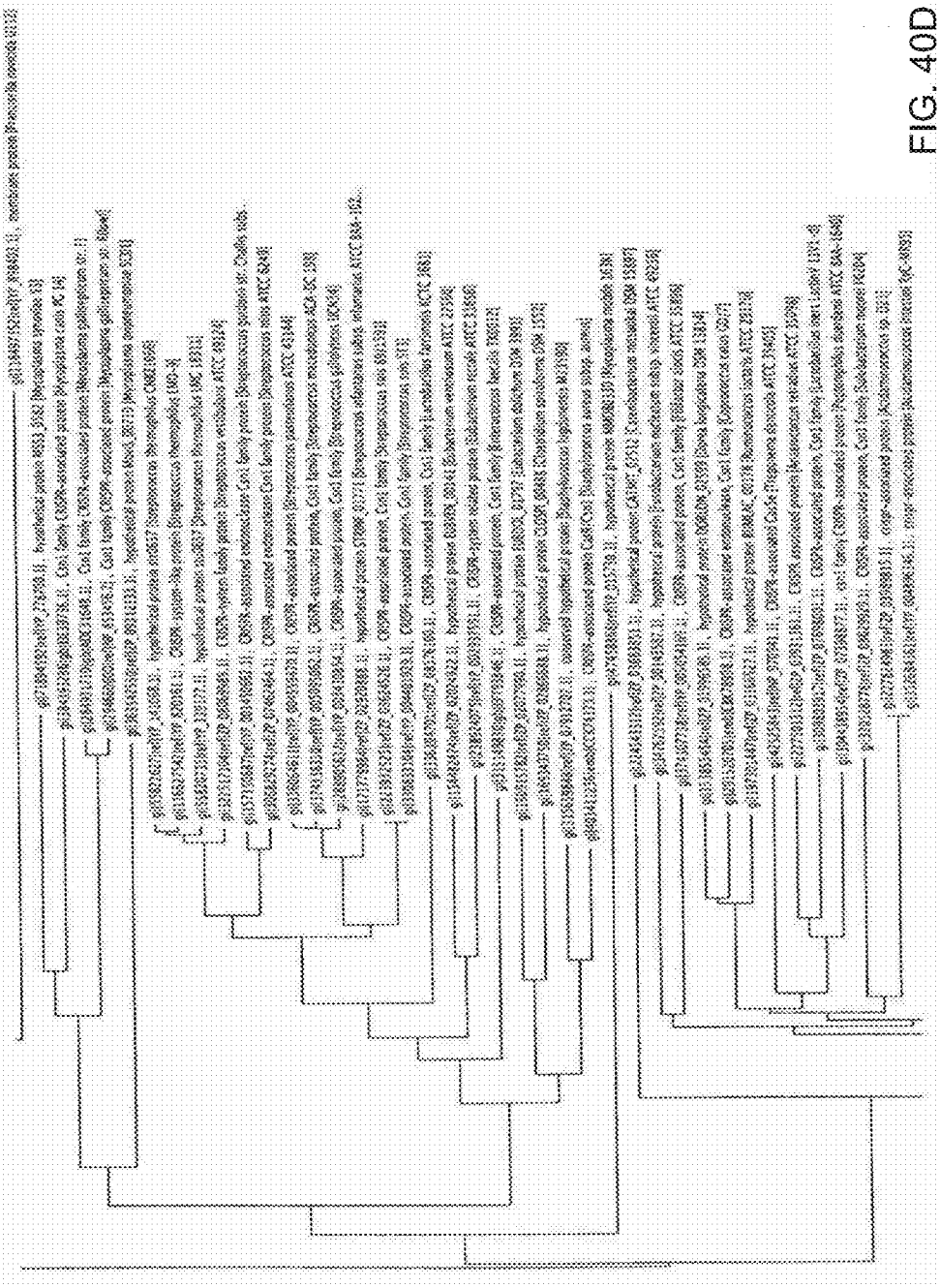


FIG. 40D

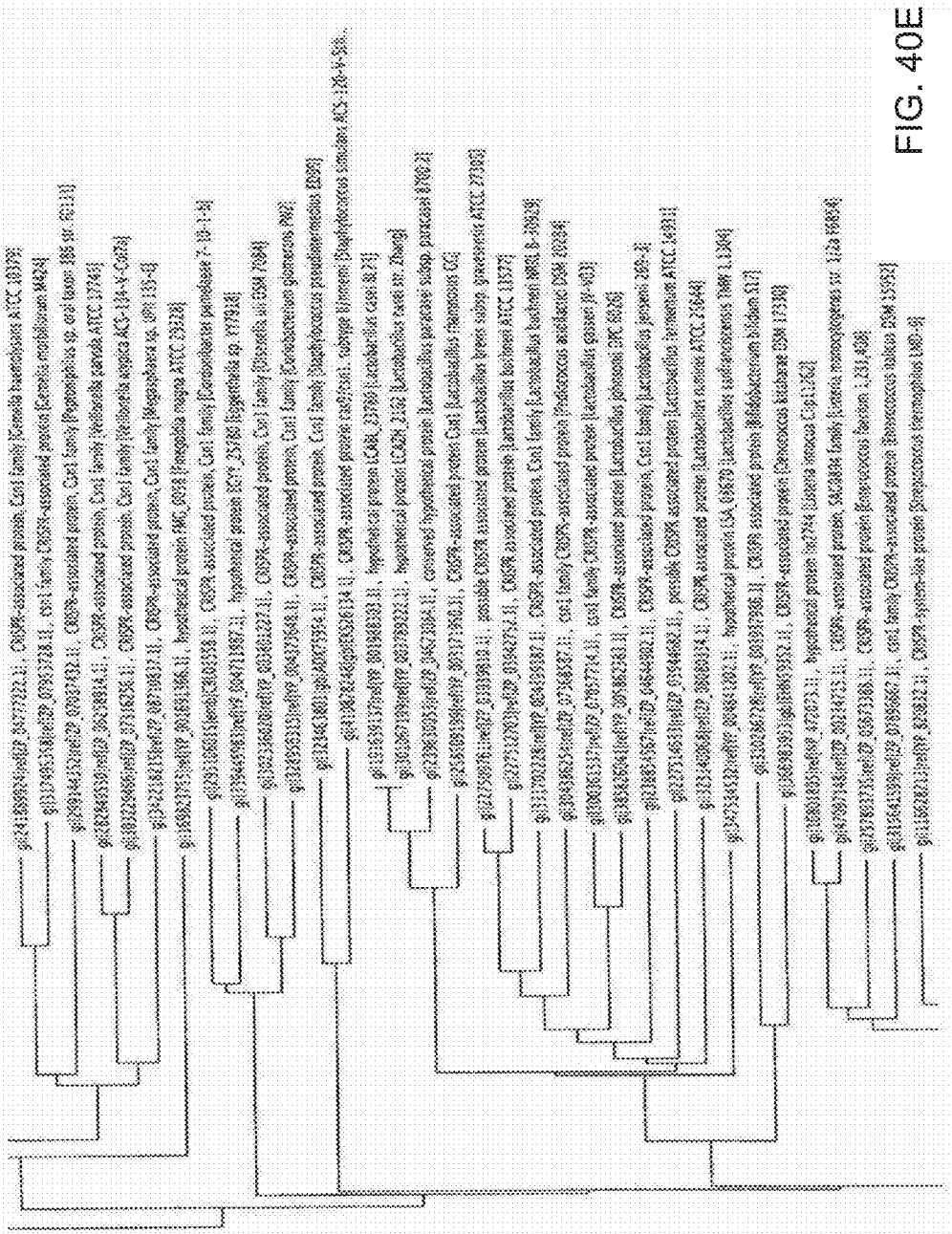


FIG. 40E

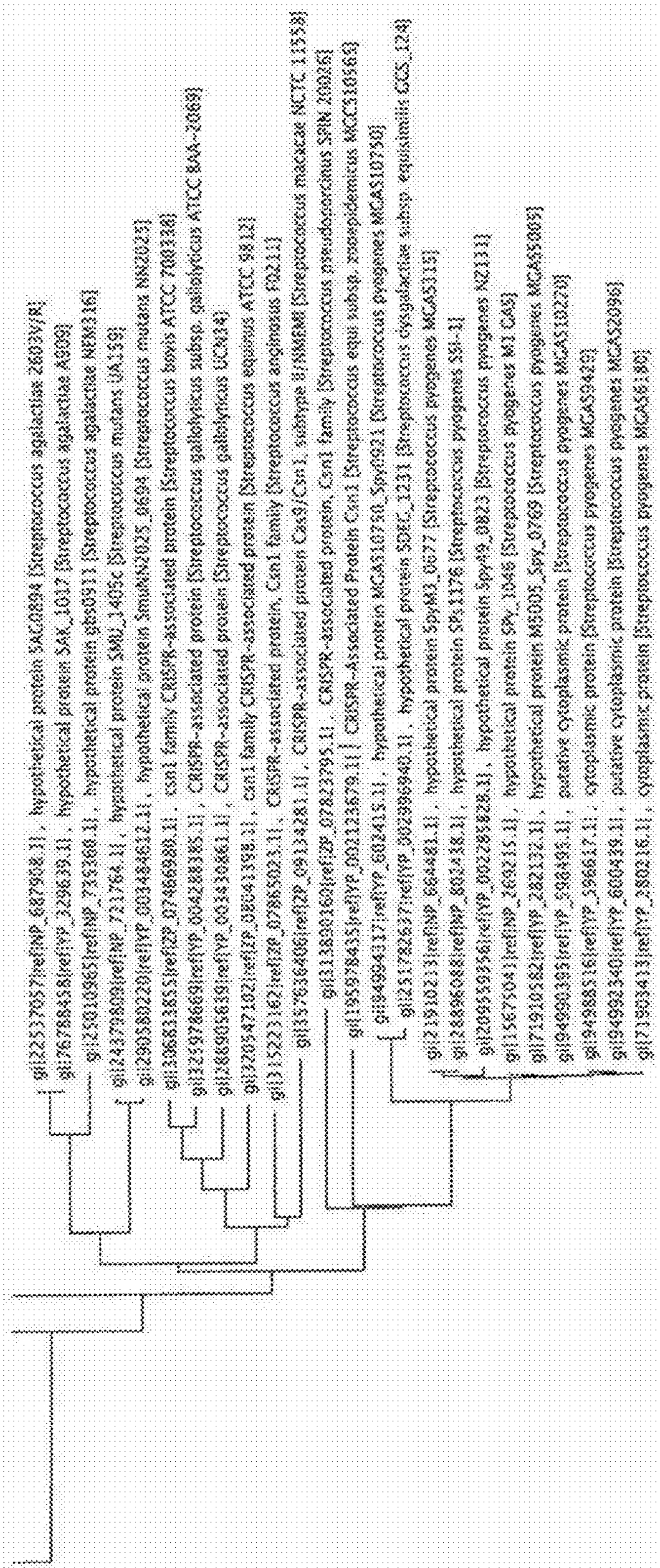


FIG. 40F

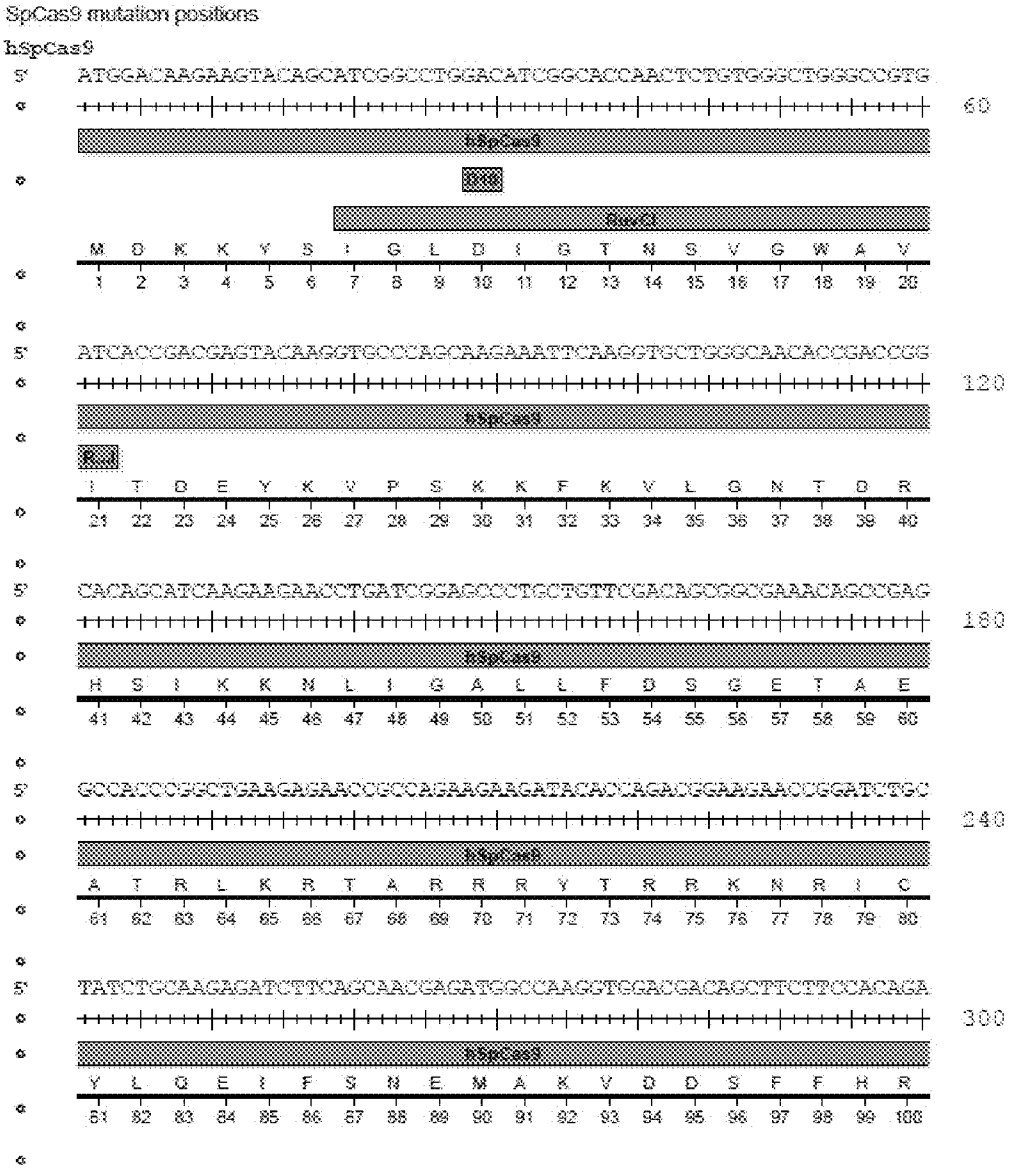

SpCas9 mutation positions
hSpCas9
5'
ATGGACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTG
60
hSpCas9
D10
Cas9_1
M D K K Y S I G L D I G T N S V G W A V
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
5'
ATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGG
120
hSpCas9
R_1
I T D E Y K V P S K K F K V L G N T D R
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
5'
CACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAG
180
hSpCas9
H S I K K N L I G A L L F D S G E T A E
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
5'
GCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGC
240
hSpCas9
A T R L K R T A R R R Y T R R K N R I C
61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80
5'
TATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGA
300
hSpCas9
Y L Q E I F S N E M A K V D D S F F H R
81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

FIG. 41A

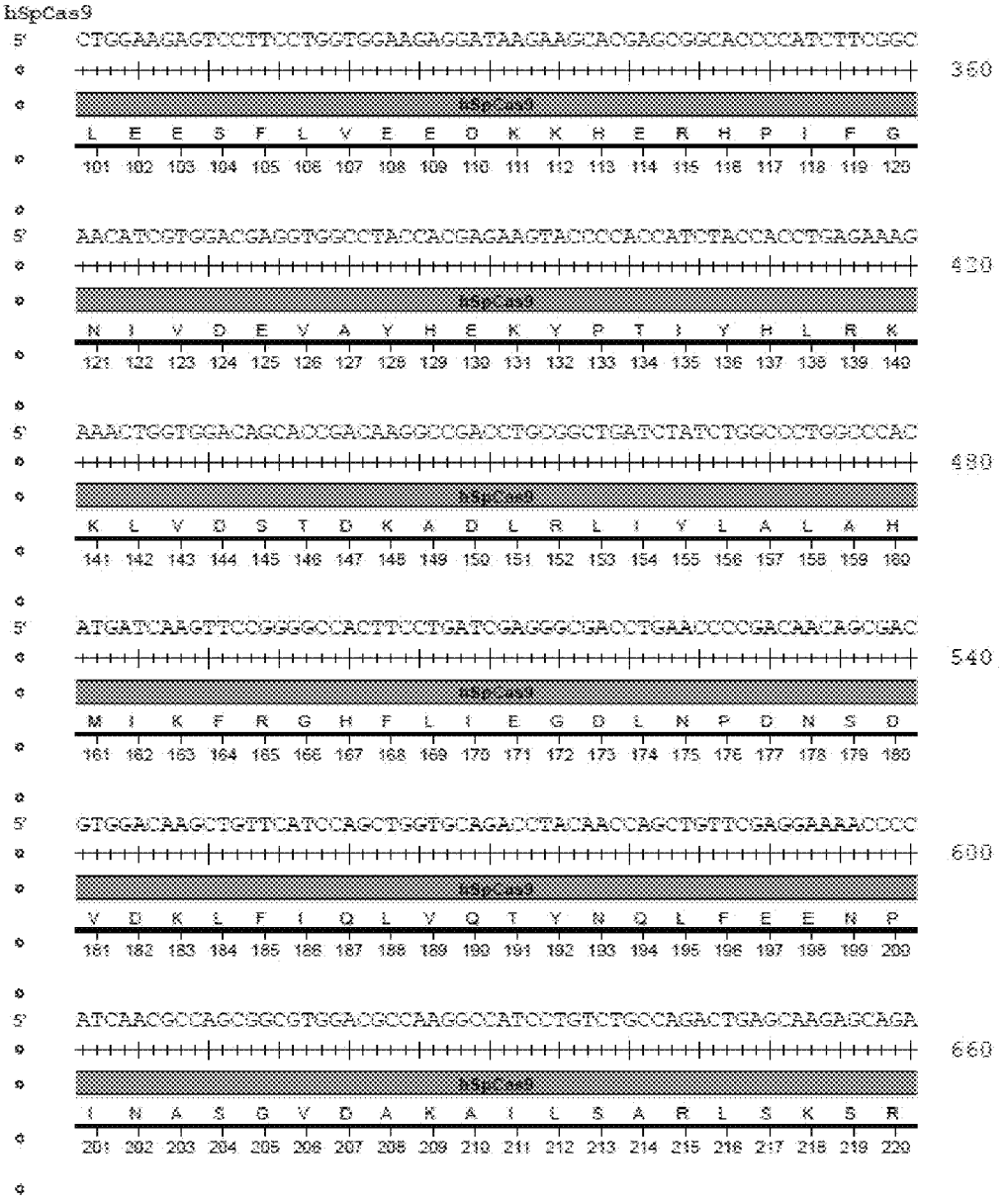

hSpCas9
5'
CTGGAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCGGC
360
hSpCas9
L E E S F L V E E D K K H E R H P I F G
101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
5'
AACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCCACCATCTACCACCTGAGAAAG
420
hSpCas9
N I V D E V A Y H E K Y P T I Y H L R K
121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140
5'
AAACTGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCAC
480
hSpCas9
K L V D S T D K A D L R L I Y L A L A H
141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160
5'
ATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTGAACCCCGACAACAGCGAC
540
hSpCas9
M I K F R G H F L I E G D L N P D N S D
161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180
5'
GTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGGAAAACCCC
600
hSpCas9
V D K L F I Q L V Q T Y N Q L F E E N P
181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200
5'
ATCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGA
660
hSpCas9
I N A S G V D A K A I L S A R L S K S R
201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220

FIG. 41B

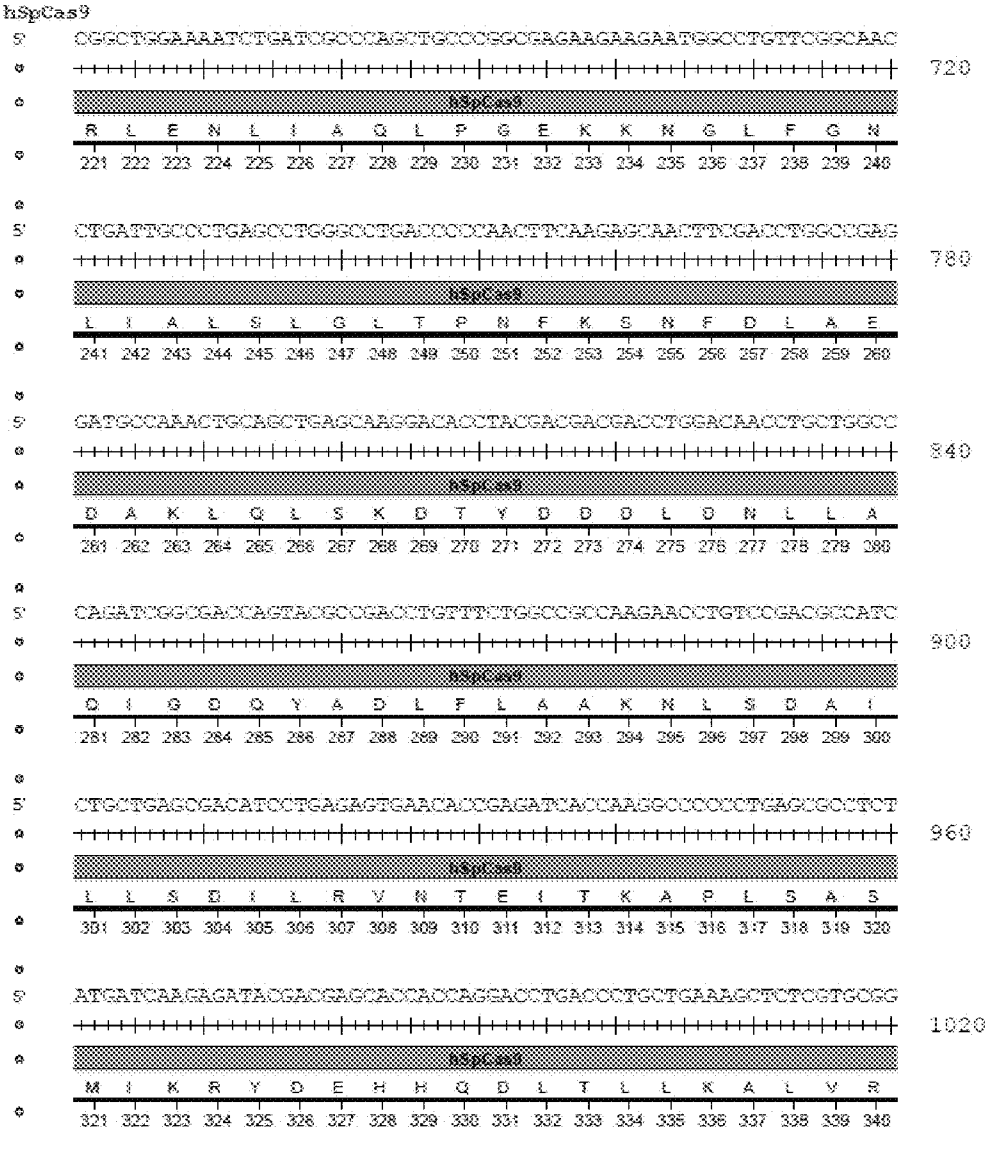


FIG. 41C

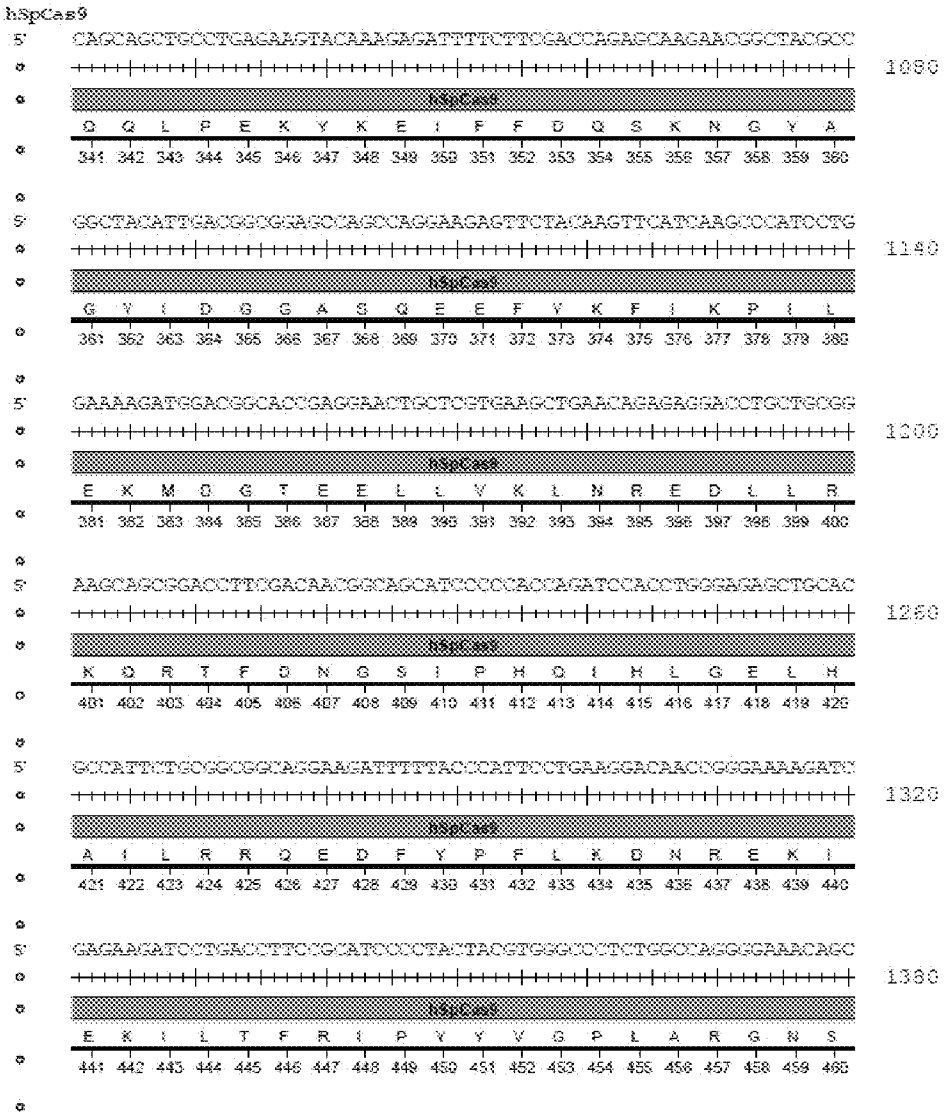

hSpCas9
5'
CAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCC
1080
hSpCas9
Q Q L P E K Y K E I F F D Q S K N G Y A
341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360
5'
GGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTG
1140
hSpCas9
G Y I D G G A S Q E E F Y K F I K P I L
361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380
5'
GAAAAGATGGACGGCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGG
1200
hSpCas9
E K M D G T E E L L V K L N R E D L L R
381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400
5'
AAGCAGCGGACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCTGGGAGAGCTGCAC
1260
hSpCas9
K Q R T F D N G S I P H Q I H L G E L H
401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420
5'
GCCATTCTGCGGCGGCAGGAAGATTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATC
1320
hSpCas9
A I L R R Q E D F Y P F L K D N R E K I
421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440
5'
GAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAGC
1380
hSpCas9
E K I L T F R I P Y Y V G P L A R G N S
441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460

FIG. 41D

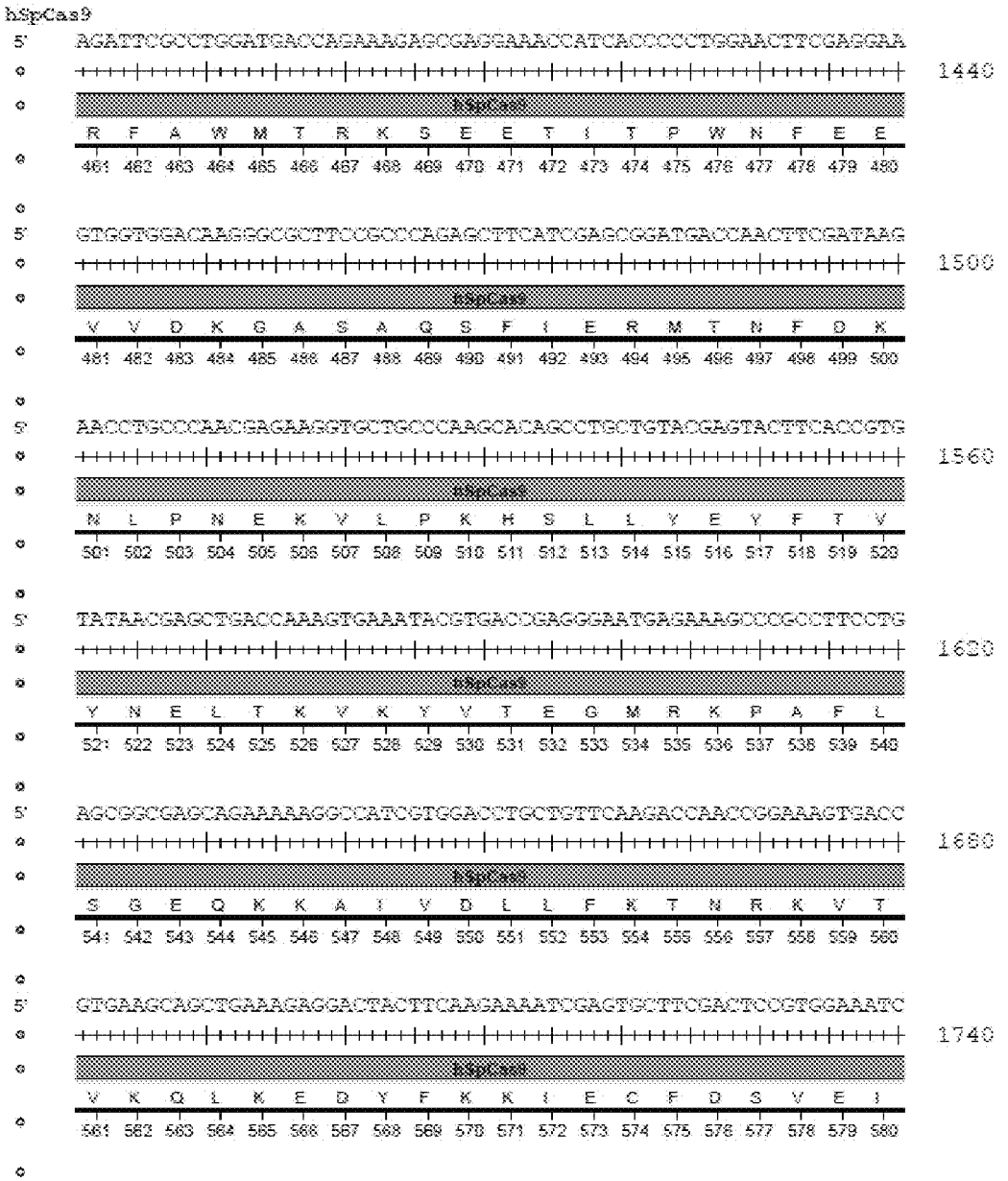

hSpCas9
5'
AGATTCGCCTGGATGACCAGAAAGAGCGAGGAAACCATCACCCCCTGGAACTTCGAGGAA
1440
hSpCas9
R F A W M T R K S E E T I T P W N F E E
461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480
5'
GTGGTGGACAAGGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAG
1500
hSpCas9
V V D K G A S A Q S F I E R M T N F D K
481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500
5'
AACCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTACGAGTACTTCACCGTG
1560
hSpCas9
N L P N E K V L P K H S L L Y E Y F T V
501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520
5'
TATAACGAGCTGACCAAAGTGAAATACGTGACCGAGGGAATGAGAAAGCCCGCCTTCCTG
1620
hSpCas9
Y N E L T K V K Y V T E G M R K P A F L
521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540
5'
AGCGGCGAGCAGAAAAAGGCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACC
1680
hSpCas9
S G E Q K K A I V D L L F K T N R K V T
541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560
5'
GTGAAGCAGCTGAAAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATC
1740
hSpCas9
V K Q L K E D Y F K K I E C F D S V E I
561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580

FIG. 41E

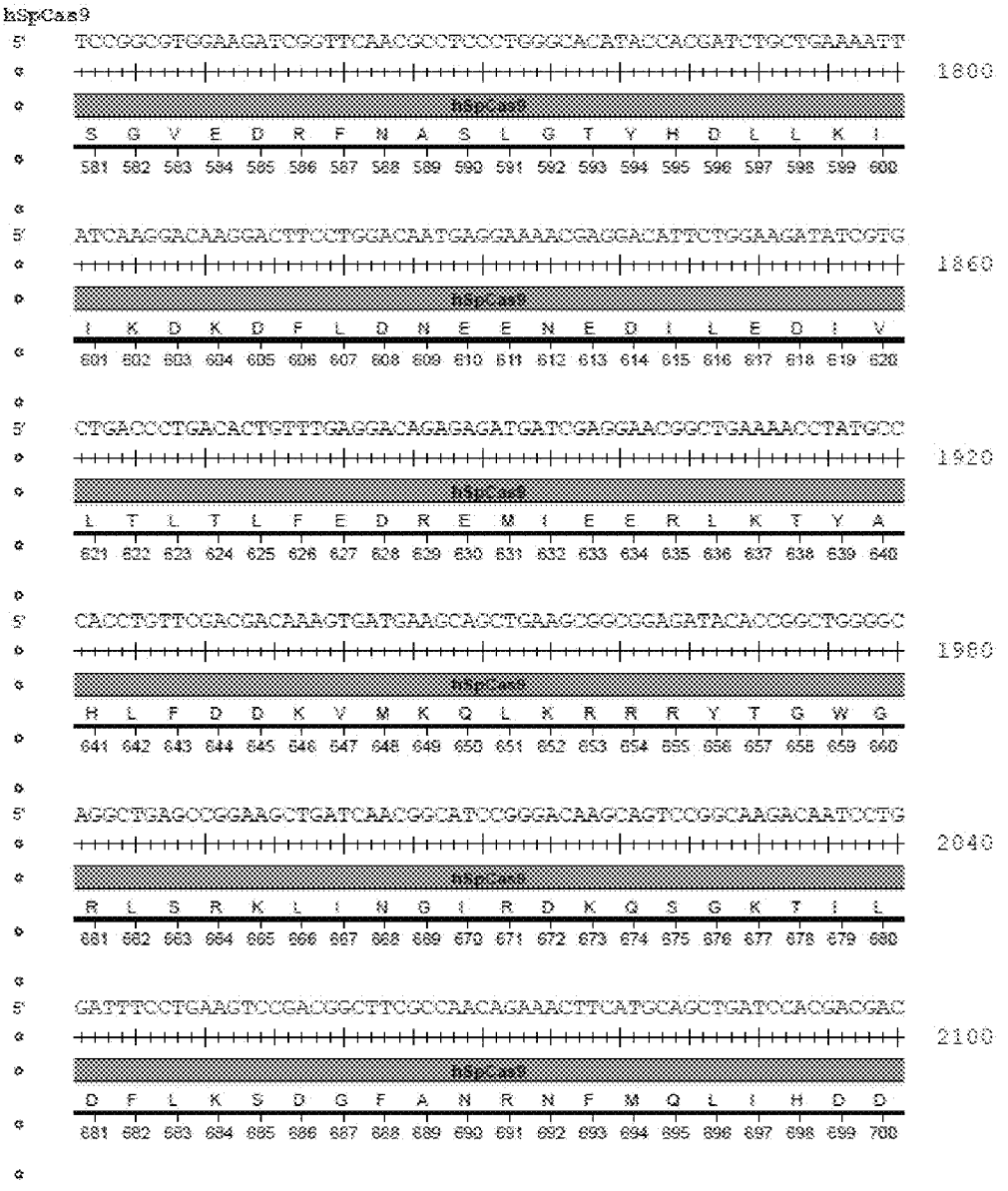

hSpCas9
5'
TCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATT
1800
hSpCas9
S G V E D R F N A S L G T Y H D L L K I
581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600
5'
ATCAAGGACAAGGACTTCCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTG
1860
hSpCas9
I K D K D F L D N E E N E D I L E D I V
601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620
5'
CTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCC
1920
hSpCas9
L T L T L F E D R E M I E E R L K T Y A
621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640
5'
CACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGGC
1980
hSpCas9
H L F D D K V M K Q L K R R R Y T G W G
641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660
5'
AGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACAATCCTG
2040
hSpCas9
R L S R K L I N G I R D K Q S G K T I L
661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680
5'
GATTTCCTGAAGTCCGACGGCTTCGCCAACAGAAACTTCATGCAGCTGATCCACGACGAC
2100
hSpCas9
D F L K S D G F A N R N F M Q L I H D D
681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700

FIG. 41F

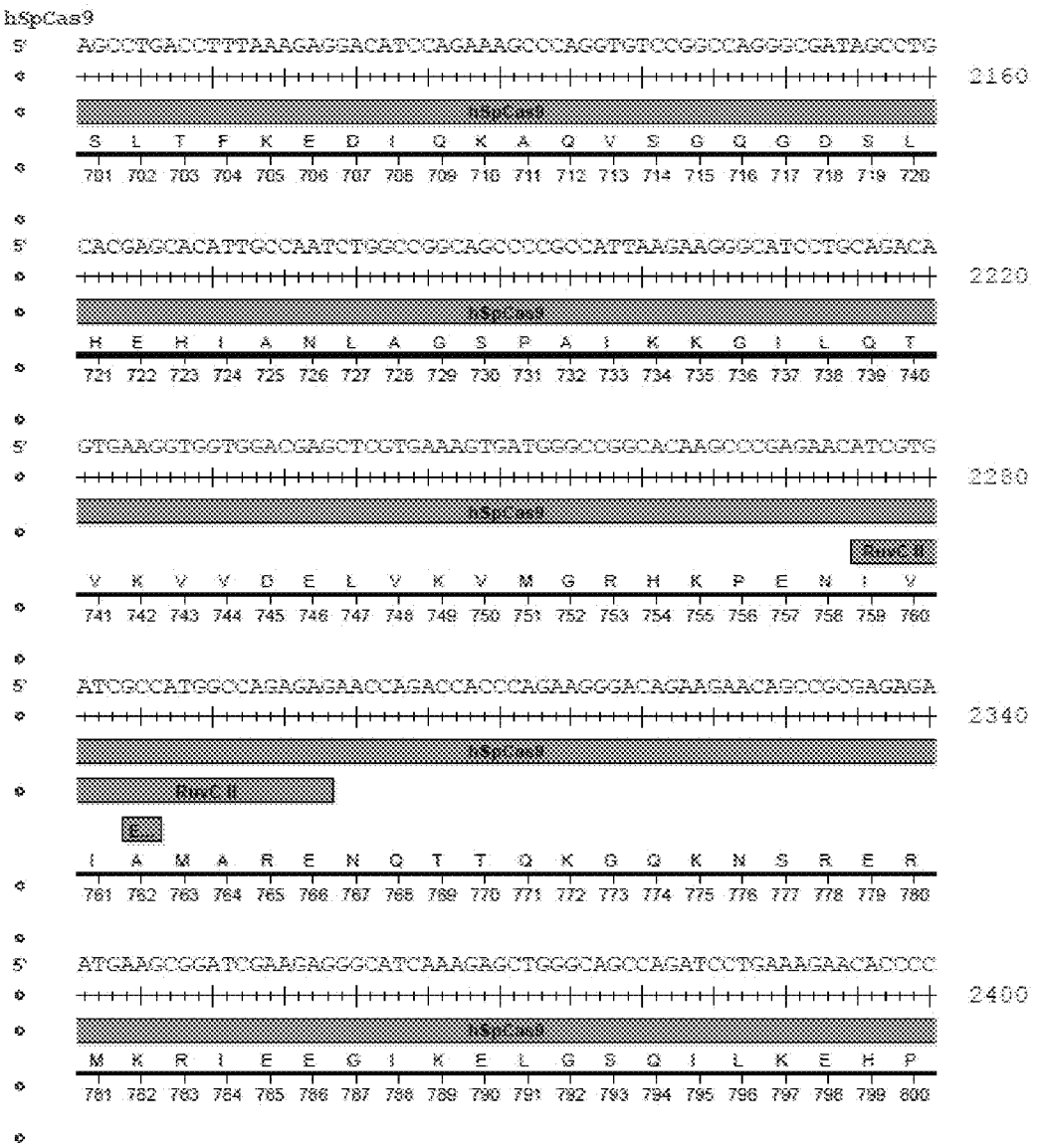

hSpCas9
5'
2160
S L T F K E D I Q K A Q V S G Q G D S L
781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800
5'
2220
H E H I A N L A G S P A I K K G I L Q T
721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740
5'
2280
V K V V D E L V K V M G R H K P E N I V
741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760
5'
2340
I A M A R E N Q T T Q K G Q K N S R E R
761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780
5'
2400
M K R I E E G I K E L G S Q I L K E H P
781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800

FIG. 41G

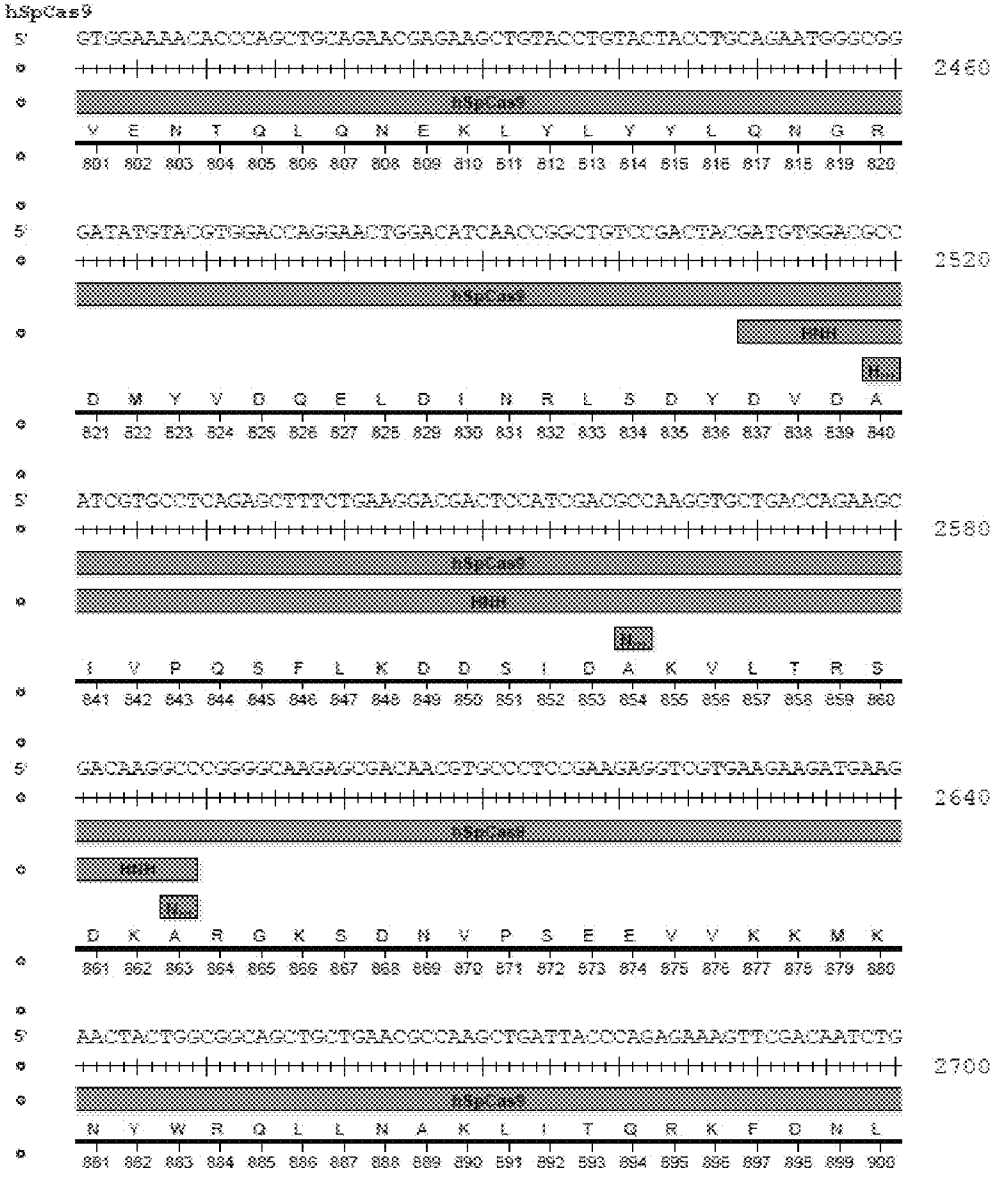

hSpCas9
5'
GTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAATGGGCGG
2460
hSpCas9
V E N T Q L Q N E K L Y L Y Y L Q N G R
801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820
5'
GATATGTACGTGGACCAGGAACTGGACATCAACCGGCTGTCCGACTACGATGTGGACGCC
2520
hSpCas9
HNH
D M Y V D Q E L D I N R L S D Y D V D A
821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840
5'
ATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCGACGCCAAGGTGCTGACCAGAAGC
2580
hSpCas9
HNH
I V P Q S F L K D D S I D A K V L T R S
841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860
5'
GACAAGGCCCGGGGCAAGAGCGACAACGTGCCCTCCGAAGAGGTCGTGAAGAAGATGAAG
2640
hSpCas9
HNH
D K A R G K S D N V P S E E V V K K M K
861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880
5'
AACTACTGGCGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTG
2700
hSpCas9
N Y W R Q L L N A K L I T Q R K F D N L
881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900

FIG. 41H

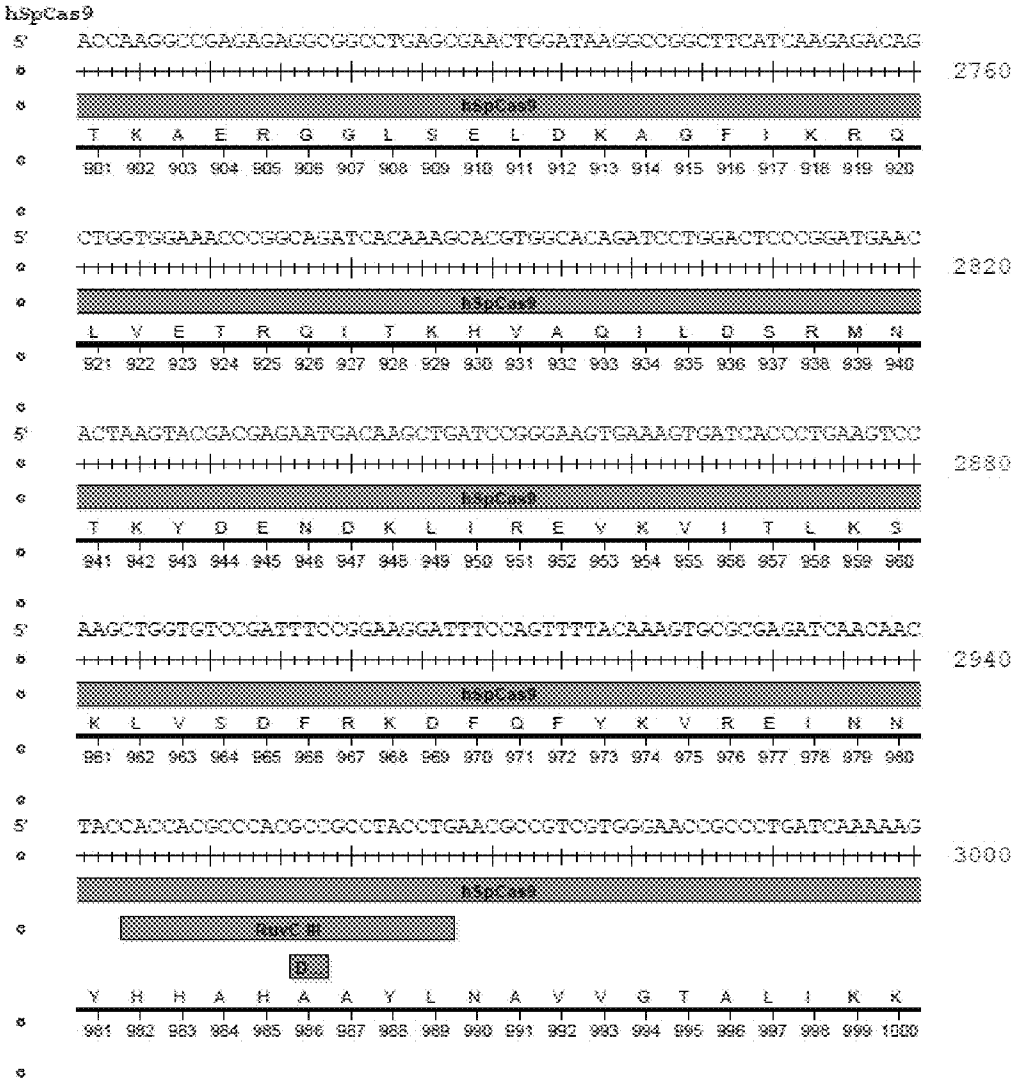

hSpCas9
5'
2760
2820
2880
2940
3000
T K A E R G G L S E L D K A G F I K R Q
L V E T R Q I T K H V A Q I L D S R M N
T K Y D E N D K L I R E V K V I T L K S
K L V S D F R K D F Q F Y K V R E I N N
Y H H A H A A Y L N A V V G T A L I K K

FIG. 41I

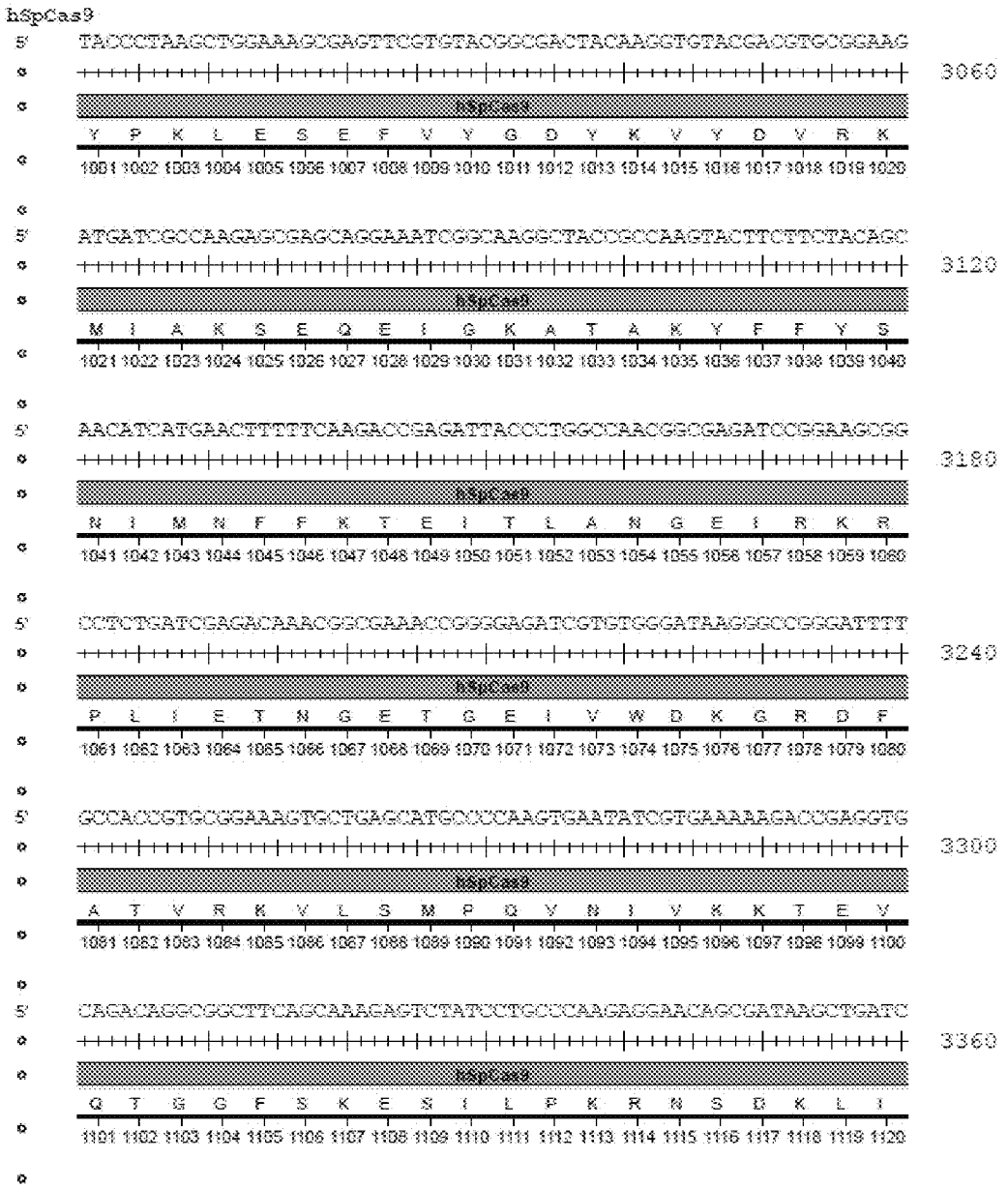

hSpCas9
5' TACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGCGGAAG 3060
hSpCas9
Y P K L E S E F V Y G D Y K V Y D V R K
1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020
5' ATGATCGCCAAGAGCGAGCAGGAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGC 3120
hSpCas9
M I A K S E Q E I G K A T A K Y F F Y S
1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040
5' AACATCATGAACTTTTTCAAGACCGAGATTACCCTGGCCAACGGCGAGATCCGGAAGCGG 3180
hSpCas9
N I M N F F K T E I T L A N G E I R K R
1041 1042 1043 1044 1045 1046 1047 1048 1049 1050 1051 1052 1053 1054 1055 1056 1057 1058 1059 1060
5' CCTCTGATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGATTTT 3240
hSpCas9
P L I E T N G E T G E I V W D K G R D F
1061 1062 1063 1064 1065 1066 1067 1068 1069 1070 1071 1072 1073 1074 1075 1076 1077 1078 1079 1080
5' GCCACCGTGCGGAAAGTGCTGAGCATGCCCCAAGTGAATATCGTGAAAAAGACCGAGGTG 3300
hSpCas9
A T V R K V L S M P Q V N I V K K T E V
1081 1082 1083 1084 1085 1086 1087 1088 1089 1090 1091 1092 1093 1094 1095 1096 1097 1098 1099 1100
5' CAGACAGGCGGCTTCAGCAAAGAGTCTATCCTGCCCAAGAGGAACAGCGATAAGCTGATC 3360
hSpCas9
Q T G G F S K E S I L P K R N S D K L I
1101 1102 1103 1104 1105 1106 1107 1108 1109 1110 1111 1112 1113 1114 1115 1116 1117 1118 1119 1120

FIG. 41J

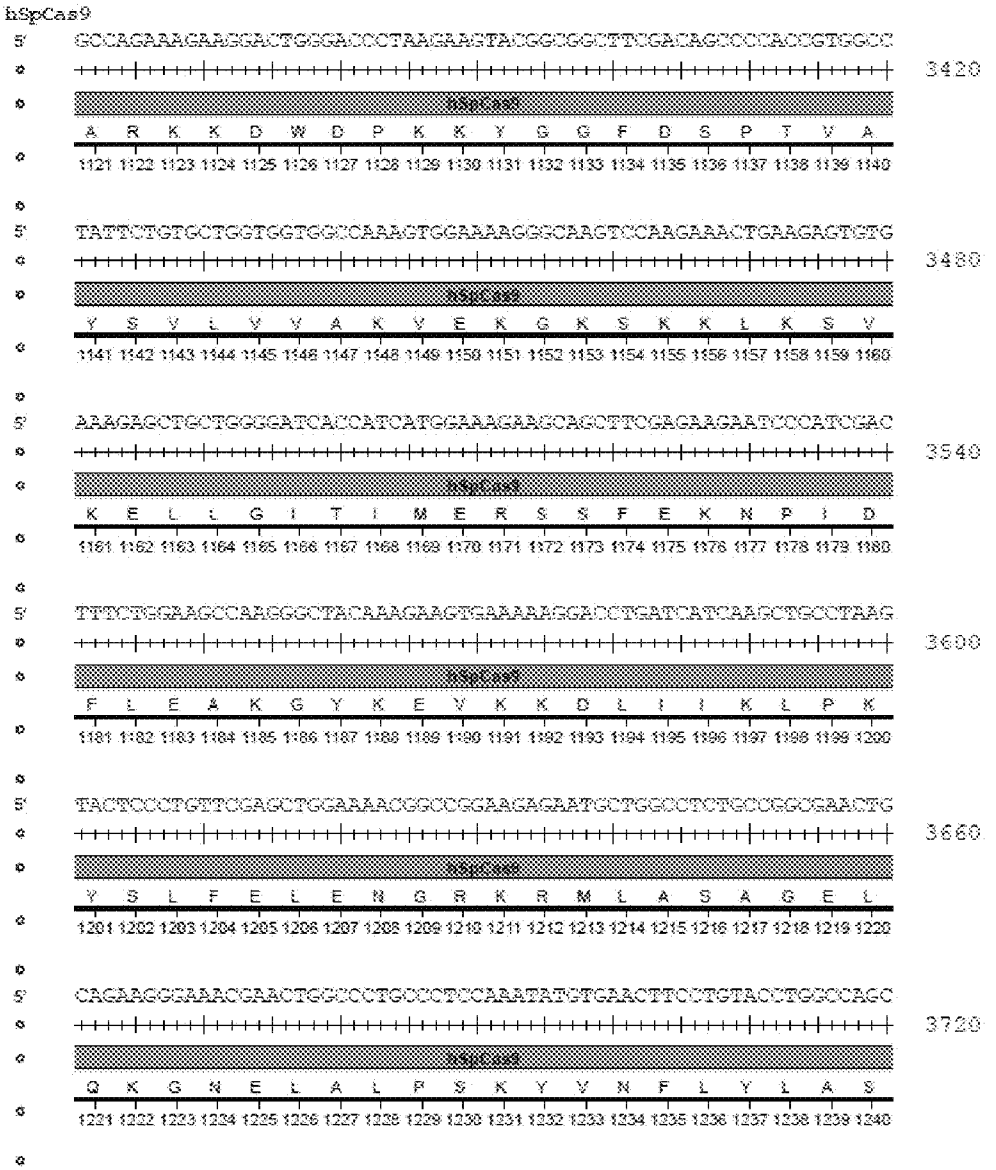

hSpCas9
5'
GCCAGAAAGAAAGGACTGGGACCCTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCC
3420
hSpCas9
A R K K D W D P K K Y G G F D S P T V A
1121 1122 1123 1124 1125 1126 1127 1128 1129 1130 1131 1132 1133 1134 1135 1136 1137 1138 1139 1140
5'
TATTCTGTGCTGGTGGTGGCCAAAGTGGAAAAGGGCAAGTCCAAGAAACTGAAGAGTGTG
3480
hSpCas9
Y S V L V V A K V E K G K S K K L K S V
1141 1142 1143 1144 1145 1146 1147 1148 1149 1150 1151 1152 1153 1154 1155 1156 1157 1158 1159 1160
5'
AAAGAGCTGCTGGGGATCACCATCATGGAAAGAAGCAGCTTCGAGAAGAATCCCATCGAC
3540
hSpCas9
K E L L G I T I M E R S S F E K N P I D
1161 1162 1163 1164 1165 1166 1167 1168 1169 1170 1171 1172 1173 1174 1175 1176 1177 1178 1179 1180
5'
TTTCTGGAAGCCAAGGGCTACAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCCTAAG
3600
hSpCas9
F L E A K G Y K E V K K D L I I K L P K
1181 1182 1183 1184 1185 1186 1187 1188 1189 1190 1191 1192 1193 1194 1195 1196 1197 1198 1199 1200
5'
TACTCCCTGTTCGAGCTGGAAAACGGCCGGAAGAGAATGCTGGCCTCTGCCGGCGAACTG
3660
hSpCas9
Y S L F E L E N G R K R M L A S A G E L
1201 1202 1203 1204 1205 1206 1207 1208 1209 1210 1211 1212 1213 1214 1215 1216 1217 1218 1219 1220
5'
CAGAAGGGAAACGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTGGCCAGC
3720
hSpCas9
Q K G N E L A L P S K Y V N F L Y L A S
1221 1222 1223 1224 1225 1226 1227 1228 1229 1230 1231 1232 1233 1234 1235 1236 1237 1238 1239 1240
5'

FIG. 41K

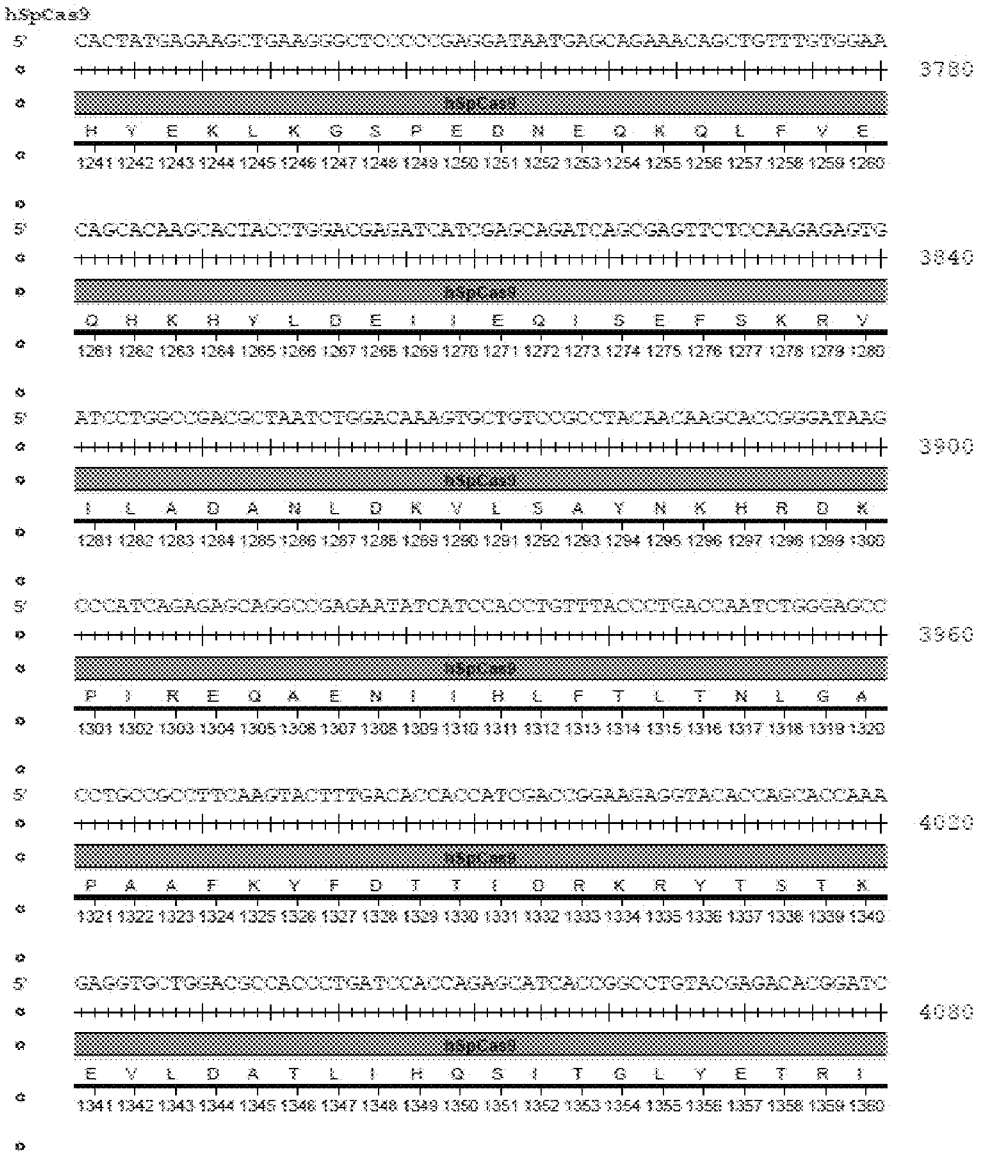

hSpCas9
5'
CACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAACAGCTGTTTGTGGAA
3780
hSpCas9
H Y E K L K G S P E D N E Q K Q L F V E
1241 1242 1243 1244 1245 1246 1247 1248 1249 1250 1251 1252 1253 1254 1255 1256 1257 1258 1259 1260
5'
CAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGTTCTCCAAGAGAGTG
3840
hSpCas9
Q H K H Y L D E I I E Q I S E F S K R V
1261 1262 1263 1264 1265 1266 1267 1268 1269 1270 1271 1272 1273 1274 1275 1276 1277 1278 1279 1280
5'
ATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACAACAAGCACCGGGATAAG
3900
hSpCas9
I L A D A N L D K V L S A Y N K H R D K
1281 1282 1283 1284 1285 1286 1287 1288 1289 1290 1291 1292 1293 1294 1295 1296 1297 1298 1299 1300
5'
CCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCC
3960
hSpCas9
P I R E Q A E N I I H L F T L T N L G A
1301 1302 1303 1304 1305 1306 1307 1308 1309 1310 1311 1312 1313 1314 1315 1316 1317 1318 1319 1320
5'
CCTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACCAAA
4020
hSpCas9
P A A F K Y F D T T I D R K R Y T S T K
1321 1322 1323 1324 1325 1326 1327 1328 1329 1330 1331 1332 1333 1334 1335 1336 1337 1338 1339 1340
5'
GAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATC
4080
hSpCas9
E V L D A T L I H Q S I T G L Y E T R I
1341 1342 1343 1344 1345 1346 1347 1348 1349 1350 1351 1352 1353 1354 1355 1356 1357 1358 1359 1360

FIG. 41L

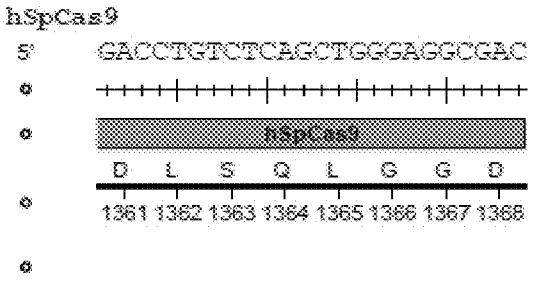

hSpCas9
5'
GACCTGTCTCAGCTGGGAGGCGAC
hSpCas9
D L S Q L G G D
1361 1362 1363 1364 1365 1366 1367 1368

FIG. 41M

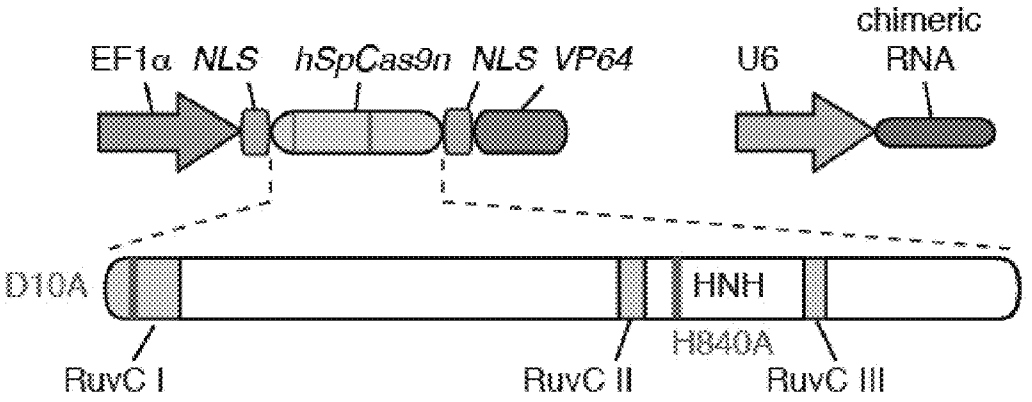

EF1α
NLS
hSpCas9n
NLS
VP64
U6
chimeric
RNA
D10A
HNH
H840A
RuvC I
RuvC II
RuvC III

FIG. 42

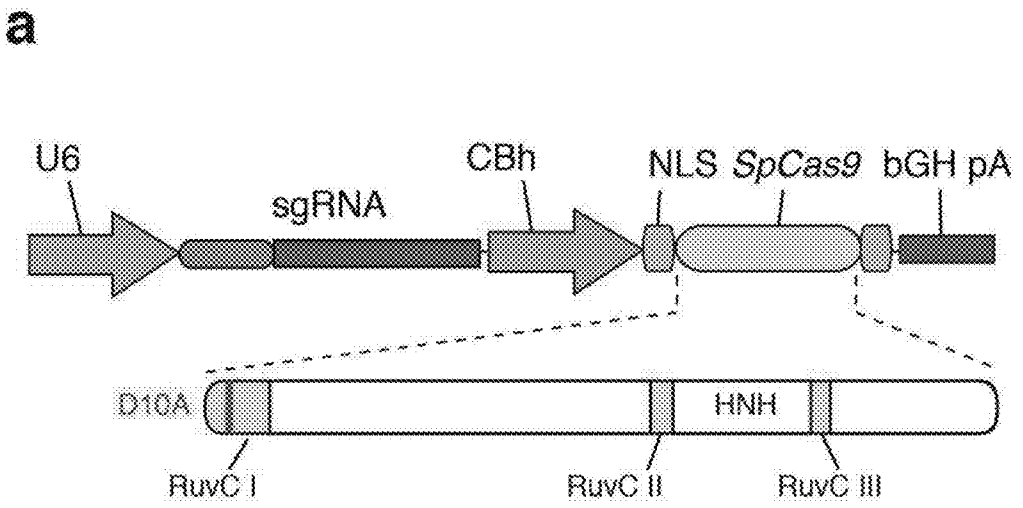

a
U6
sgRNA
CBh
NLS
SpCas9
bGH pA
D10A
HNH
RuvC I
RuvC II
RuvC III

FIG. 43A

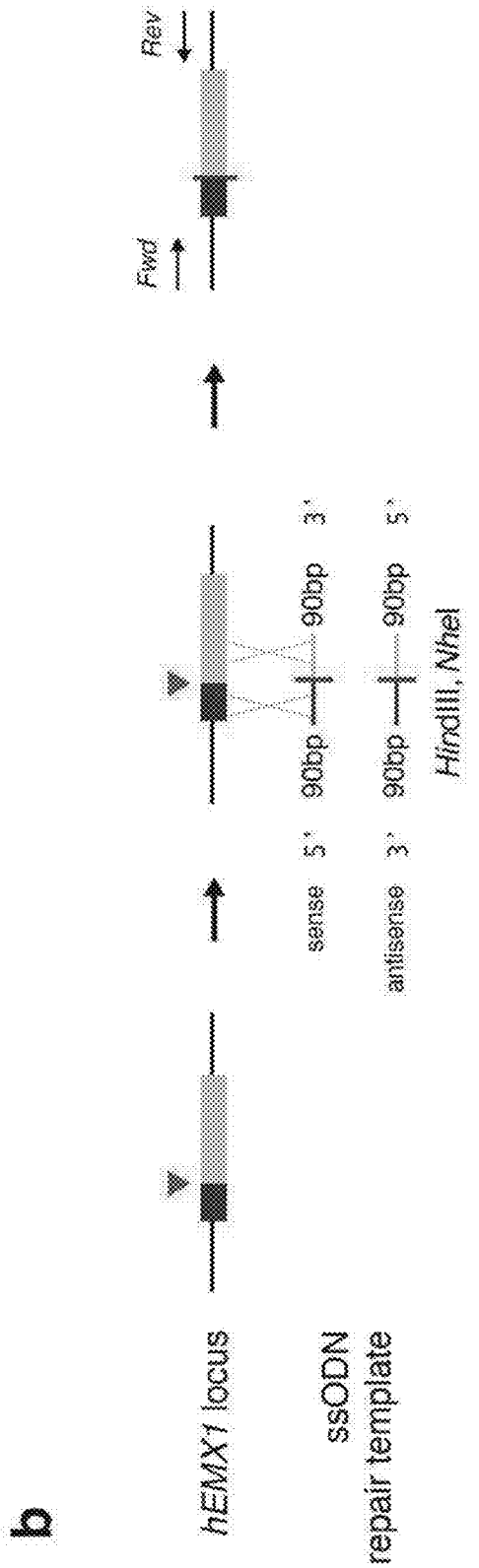

b
hEMX1 locus
ssODN
repair template
Fwd
Rev
sense
5'
90bp
3'
antisense
3'
90bp
5'
HindIII, NheI

FIG. 43B

C
hEMX1 locus
genomic target
PAM
5'- ...CAGAAGAAGAAGGGC....CCAATGGGGAGGACATCGATGTCACCTCCAATGACTAGGGTGGTGGGCAAC....CTCTGGCCACTCCCT...-3'
3'- ...GTCTTCTTCTTCCCG....GGTTACCCCTCCTGTAGCTACAGTGGAGGTTACTGATCCCACCACCCGTTG....GAGACCGGTGAGGGA...-5'
sense 5'- CAGAAGAAGAAGGGC....ACATCGATGTCACCTCCAATGACAAGCTTGCTAGCGGTGGGCAACCACAAAC....CTCTGGCCACTCCCT -3'
HindIII NheI
antisense 3'- GTCTTCTTCTTCCCG....TGTAGCTACAGTGGAGGTTACTGTTCGAACGATCGCCACCCGTTGGTGTTTG....GAGACCGGTGAGGGA -5'
HindIII NheI

FIG. 43C

d

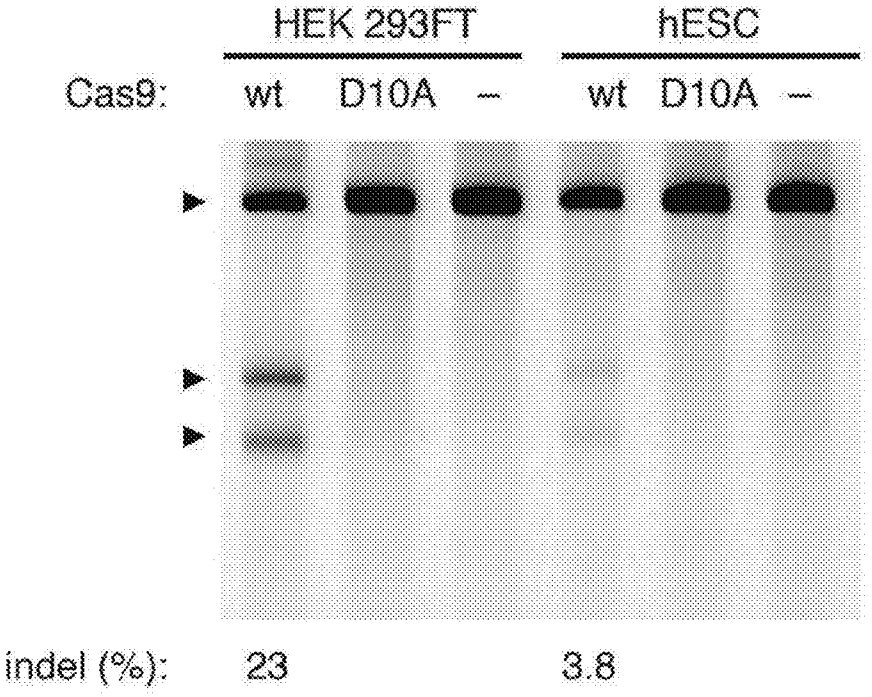


FIG. 43D

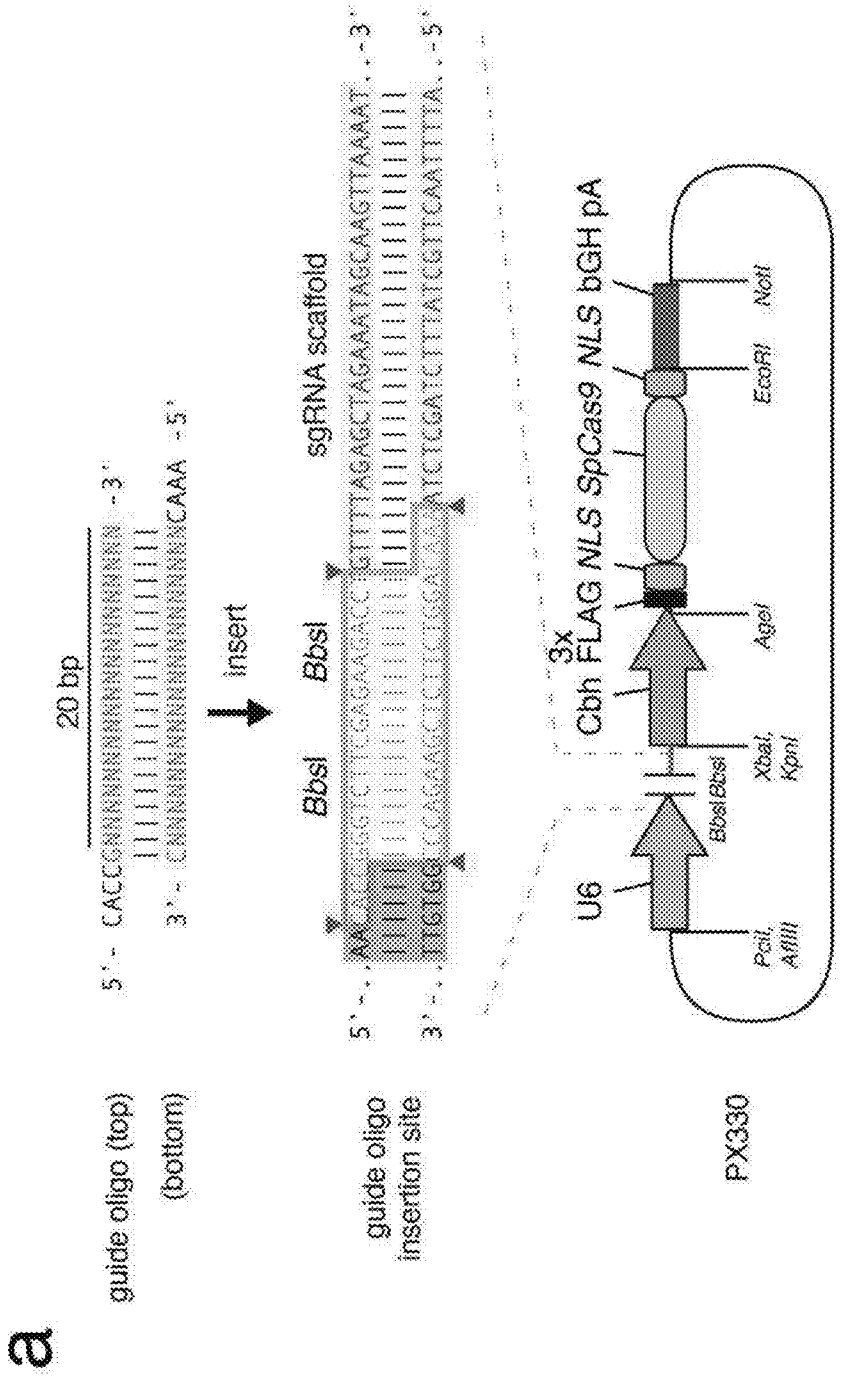

a
guide oligo (top)
(bottom)
20 bp
5' - CACC
3' - CAAA - 5'
insert
guide oligo insertion site
BbsI
BbsI
sgRNA scaffold
5' -
3' -
PX330
U6
BbsI BbsI
XbaI, KpnI
AgeI
3x Cbh FLAG NLS SpCas9 NLS bGH pA
EcoRI
NotI
PciI, AflIII

FIG. 44A

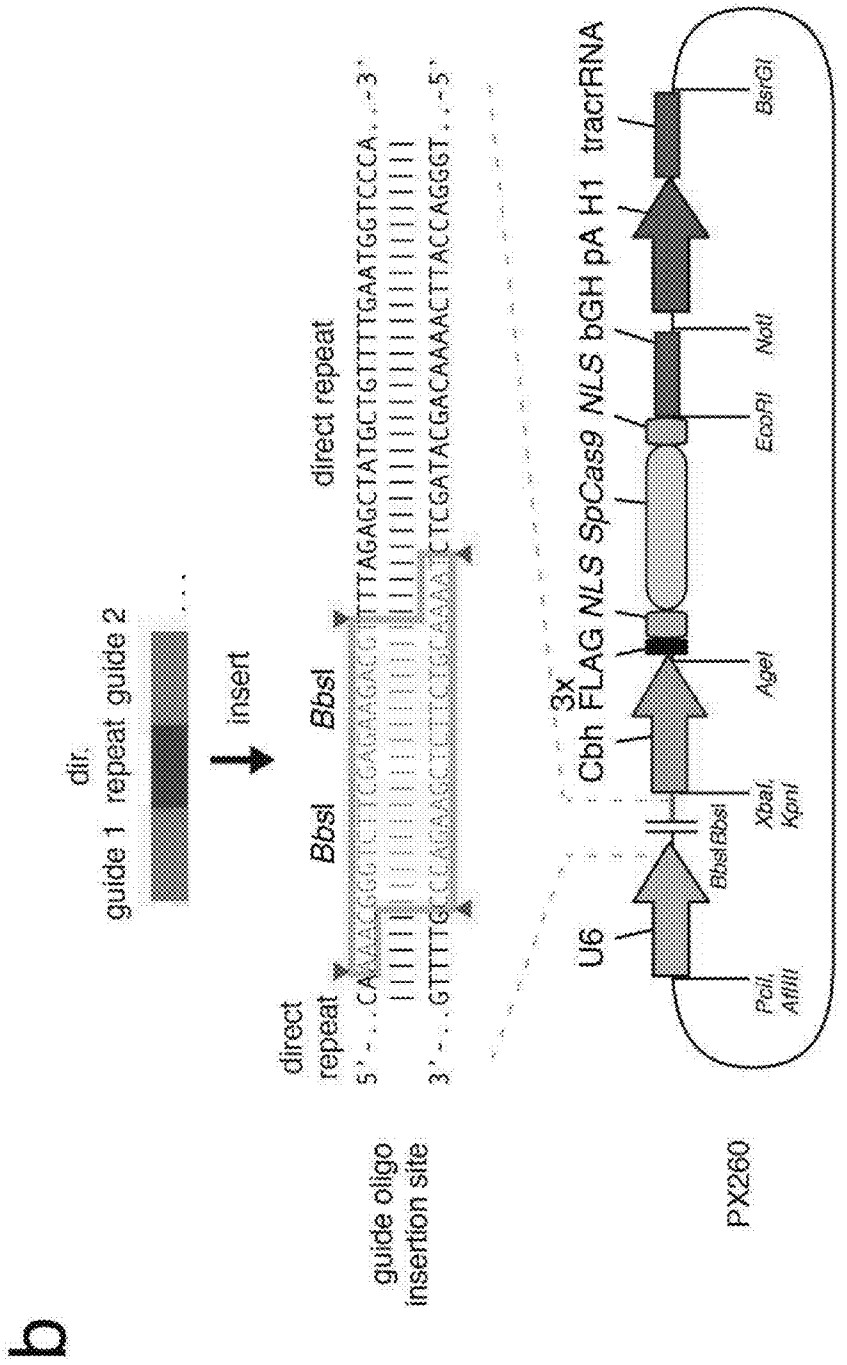

b
dir.
guide 1 repeat guide 2
insert
guide oligo
insertion site
direct
repeat
BbsI
BbsI
direct repeat
5' - . . CA
GTTTTG
TTTAGAGCTATGCTGTTTTGAATGGTCCCA . . -3'
3' - . . GTTTTG
CTCGATACGACAAAACTTACCAGGGT . . -5'
PX260
U6
3x
Cbh FLAG NLS SpCas9 NLS bGH pA H1 tracrRNA
PciI,
AflIII
BbsI BbsI
XbaI,
KpnI
AgeI
EcoRI
NotI
BsrGI

FIG. 44B

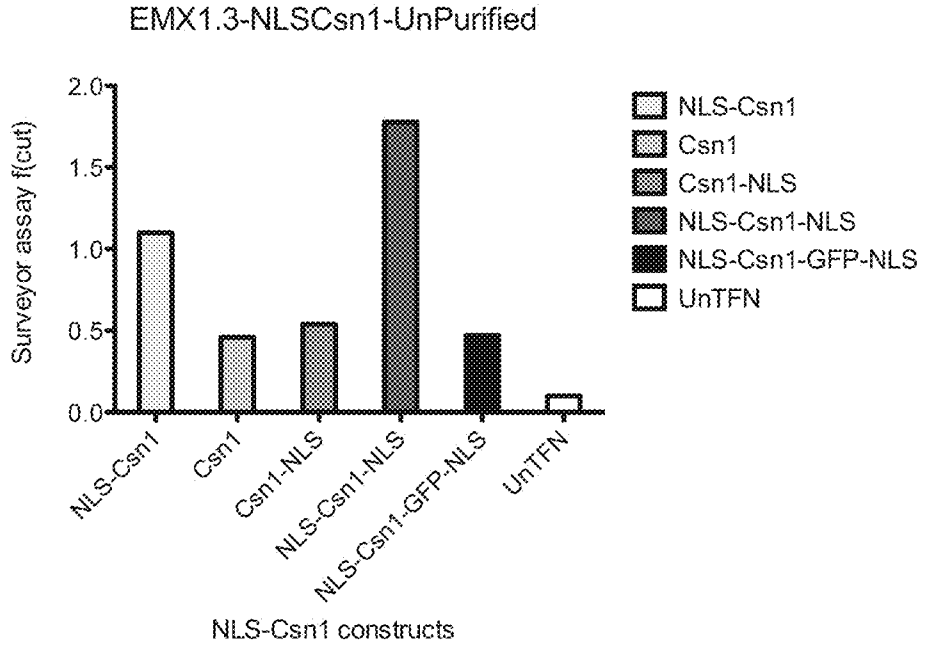

EMX1.3-NLSCsn1-UnPurified
Surveyor assay f(cut)
2.0
1.5
1.0
0.5
0.0
NLS-Csn1
Csn1
Csn1-NLS
NLS-Csn1-NLS
NLS-Csn1-GFP-NLS
UnTFN
NLS-Csn1 constructs

FIG. 45

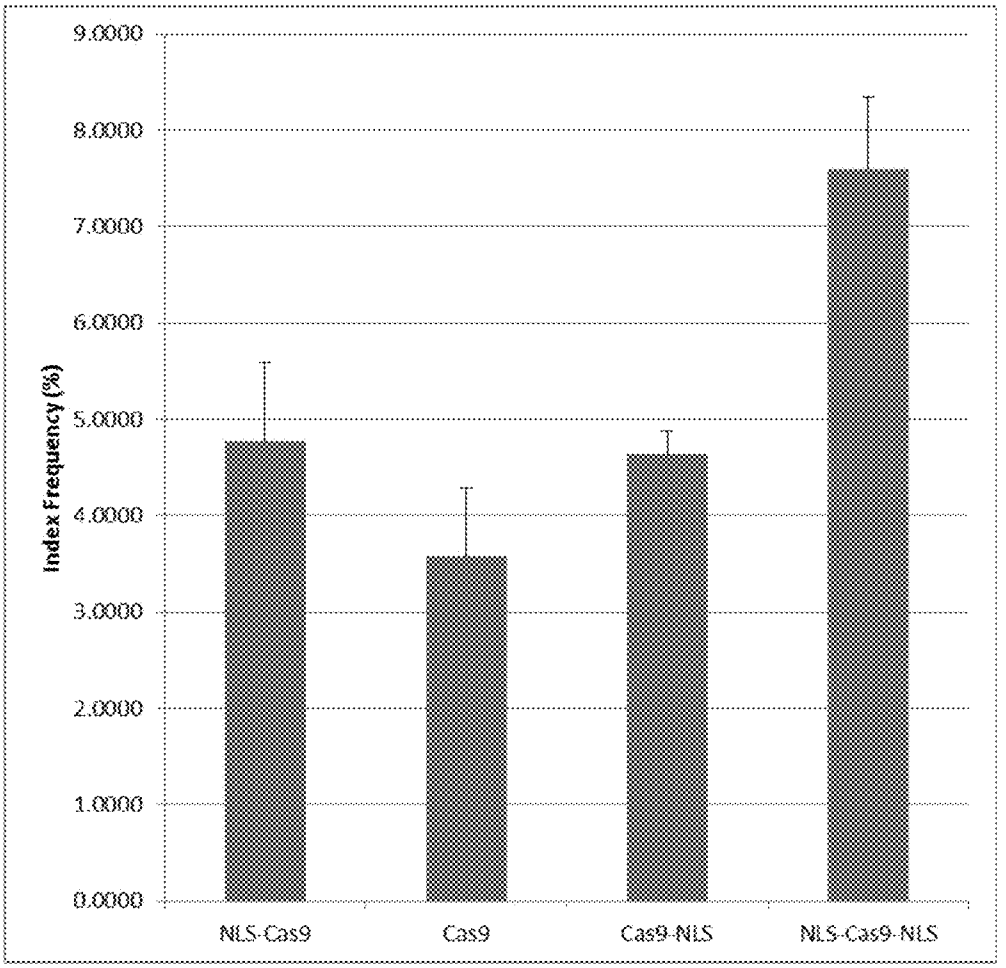

9.0000
8.0000
7.0000
6.0000
5.0000
4.0000
3.0000
2.0000
1.0000
0.0000
Index Frequency (%)
NLS-Cas9
Cas9
Cas9-NLS
NLS-Cas9-NLS

FIG. 46

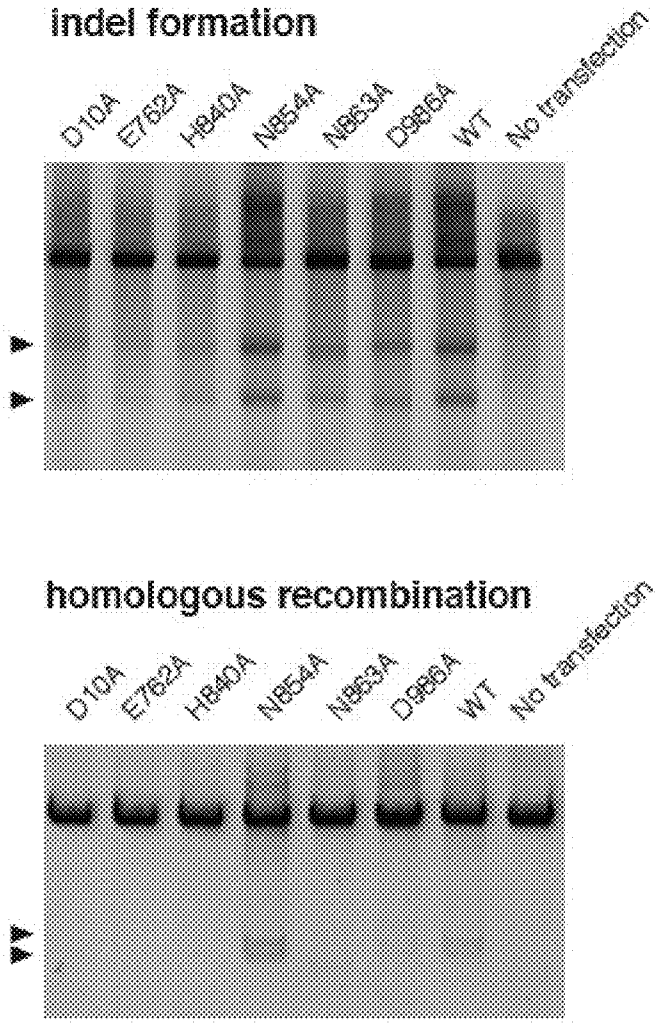
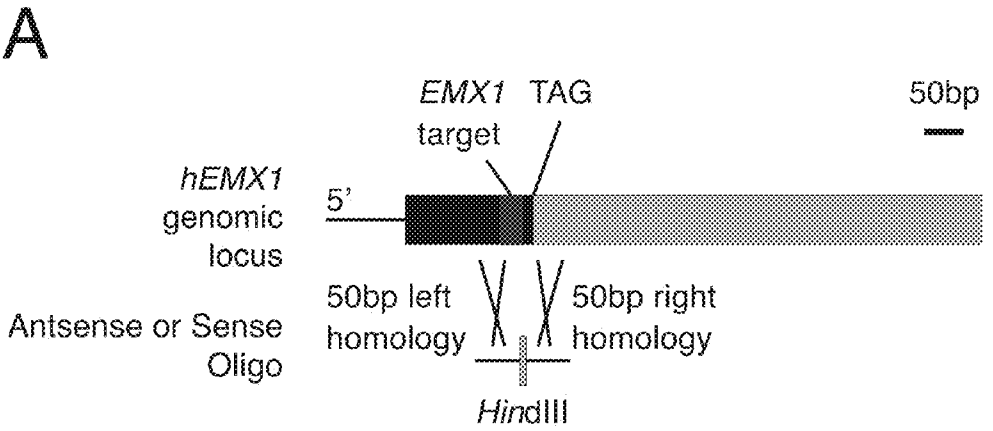

indel formation
D10A
E762A
H840A
N854A
N863A
D986A
WT
No transfection
homologous recombination
D10A
E762A
H840A
N854A
N863A
D986A
WT
No transfection

FIG. 47


A
EMX1 target
TAG
50bp
hEMX1 genomic locus
5'
50bp left homology
50bp right homology
Antsense or Sense Oligo
HindIII

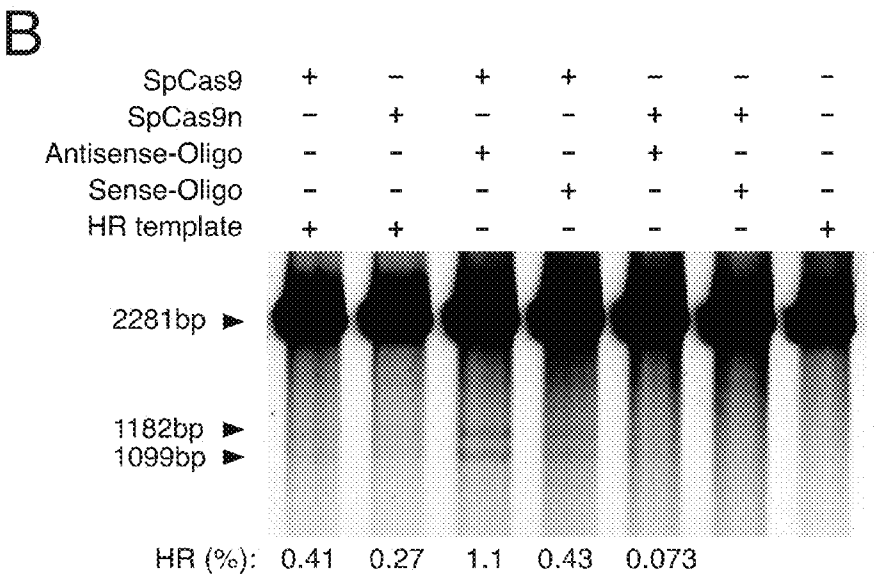

B
SpCas9 + − + + − − −
SpCas9n − + − − + + −
Antisense-Oligo − − + − + − −
Sense-Oligo − − − + − + −
HR template + + − − − − +
2281bp
1182bp
1099bp
HR (%): 0.41 0.27 1.1 0.43 0.073

FIG. 48

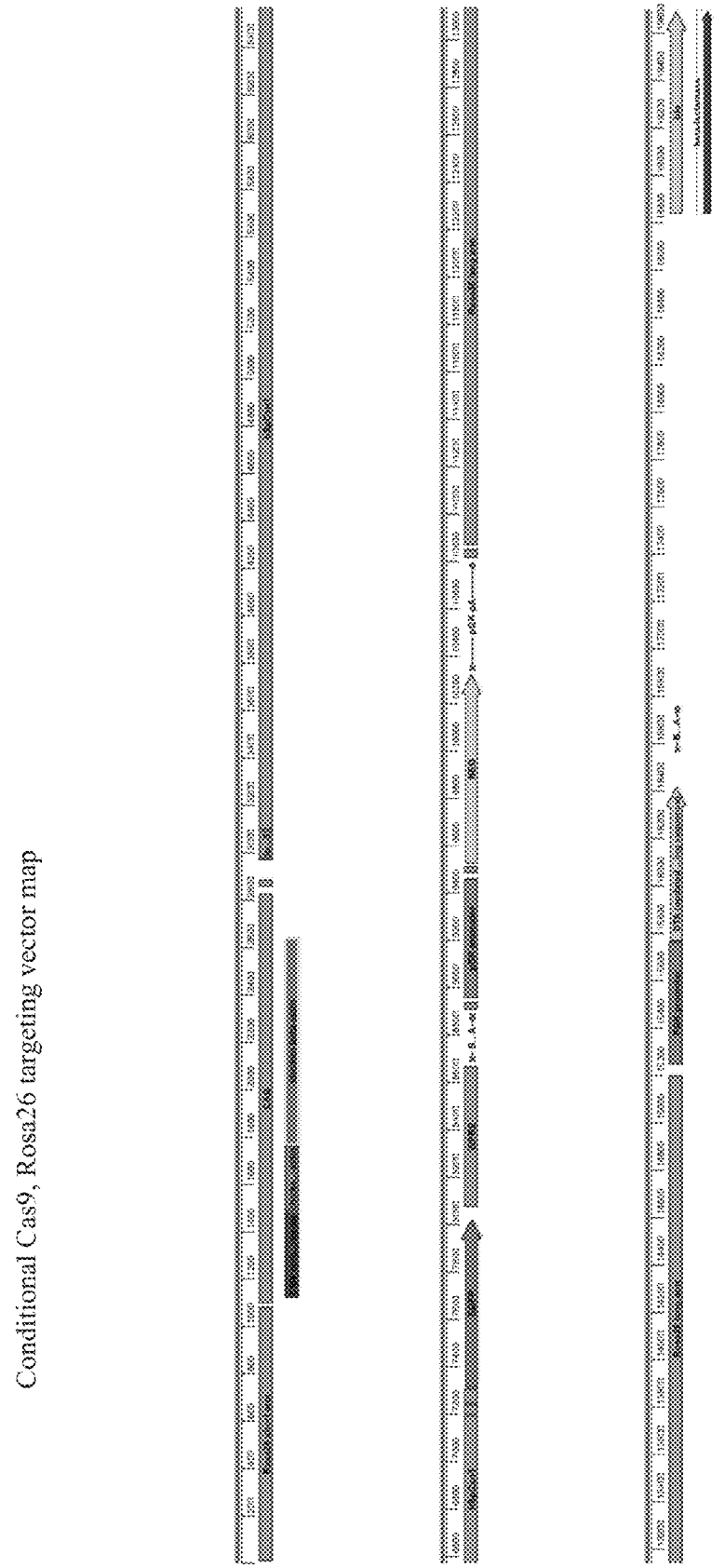


FIG. 49A

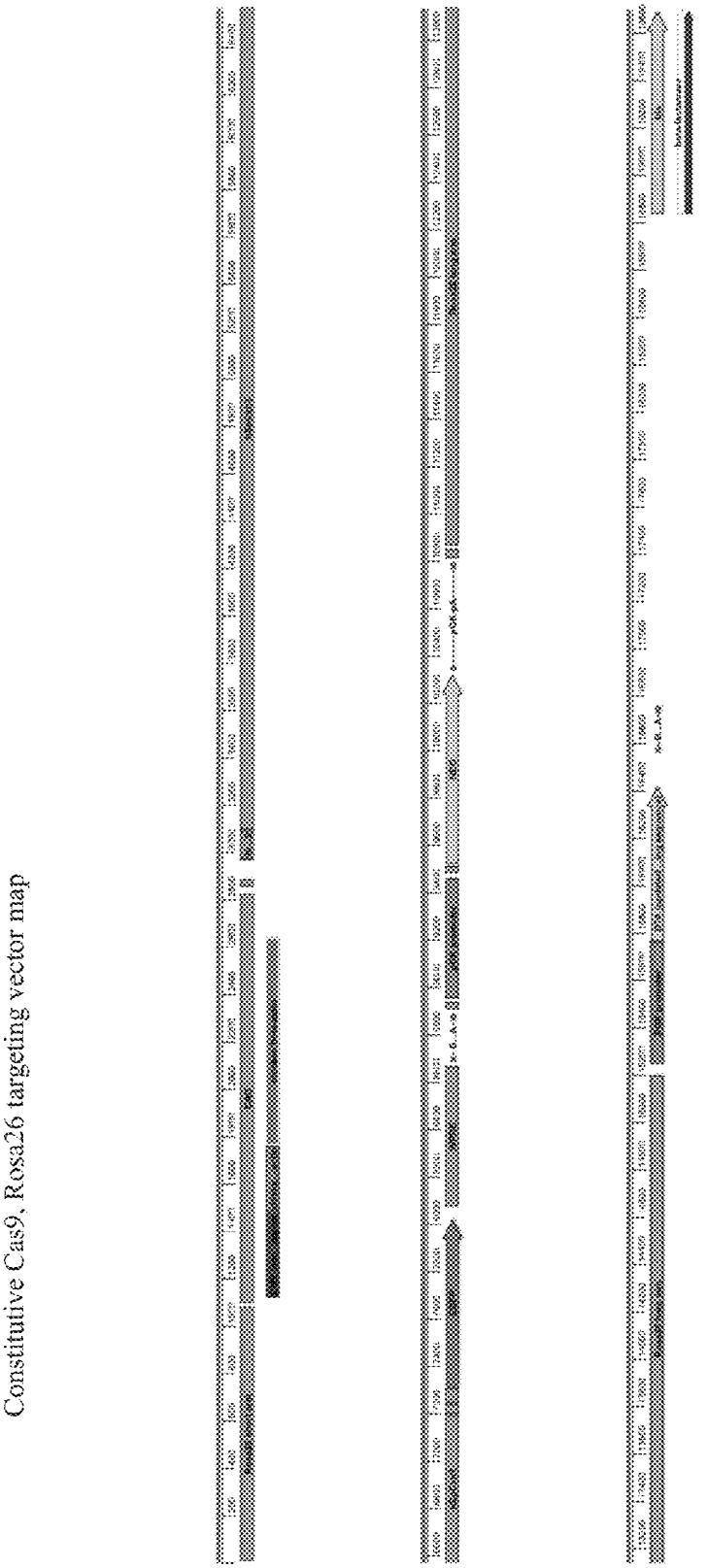


FIG. 49B

pCAG

ccgtttaaacaattctgcaggaatctagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataactt
acggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaataggga
ctttccattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgcccccctattgac
gtcaatgacggtaaatggcccgcctggcattatgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatcgc
tattaccatggtcgaggtgagccccacgttctgcttcactctcccccatctccccccctcccacccccaatttgtatttatttatttttttaattattt
gtgcagcgatgggggcgggggggggggggggcgcgcgccaggcggggcggggcggggcgaggggcggggcggggcgaggc
ggagaggtgcggcggcagccaatcagagcggcgcgctccgaaagtttccttttatggcgaggcggcggcggcggcggccctataaaaa
gcgaagcgcgcggcgggcggaAGTCGCTGCGctgccttcgccccgtgccccgctccgccgccgcctcgcgccgcccgcc
ccggctctgactgaccgcgttactcccacaggtgagcgggcgggacggcccttctcctccgggctgtaattagcgcttggtttaatgacggc
ttgtttcttttctgtggctgcgtgaaagccttgagggctccgggagggccctttgtgcgggggggagcggctcgggggggtgcgtgcgtgtgt
gtgtgcgtgggggagcgccgcgtgcggctccgcgctgcccggcggctgtgagcgctgcgggcgcggcgcgggggctttgtgcgctccgc
agtgtgcgcgaggggagcgcggccgggggcggtgccccgcggtgcgggggggggctgcgagggggaacaaaggctgcgtgcggggt
gtgtgcgtgggggggtgagcagggggtgtgggcgcgtcggtcgggctgcaaccccccctgcaccccccctccccgagttgctgagcacg
gcccggcttcgggtgcgggggctccgtacgggggcgtggcgcggggctcgccgtgccgggcgggggggtggcggcaggtgggggtgcc
gggcgggggcgggggccgcctcgggccgggggagggctcgggggggaggggcgcggcggccccccggagcgccggcggctgtcgaggcg
cggcgagccgcagccattgccttttatggtaatcgtgcgagagggcgcagggacttcctttgtcccaaatctgtgcggagccgaaatctgg
gaggcgccgccgcaccccctctagcgggcgcgggggcgaagcggtgcggcgccggcaggaaggaaatgggcggggagggccttcgt
gcgtcgccgcgccgccgtcccctctccctctccagcctcgggggctgtccgcgggggggacggctgccttcgggggggacggggcaggg
cggggttcggcttctggcgtgtgaccggcggctctagagcctctgctaaccatgttcatgccttcttctttttcctacagctcctgggcaacgtg
ctggttattgtgctgtctcatcattttggcaaa

NLS-Cas9-NLS

ATGGACTATAAGGACCACGACGGAGACTACAAGGATCATGATATTGATTACAAAGA
CGATGACGATAAGATGGCCCCAAAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCC
CAGCAGCCGACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGGGC
TGGGCCGTGATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGG
CAACACCGACCGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACA
GCGGCGAAACAGCCGAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACAC
CAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGCAACGAGATGGCCA
AGGTGGACGACAGCTTCTTCCACAGACTGGAAGAGTCCTTCCTGGTGGAAGAGGAT
AAGAAGCACGAGCGGCACCCCATCTTCGGCAACATCGTGGACGAGGTGGCCTACCA
CGAGAAGTACCCCACCATCTACCACCTGAGAAAGAAACTGGTGGACAGCACCGACA
AGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCACATGATCAAGTTCCGGGGCC
ACTTCCTGATCGAGGGCGACCTGAACCCCGACAACAGCGACGTGGACAAGCTGTTC

FIG. 50A

ATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGGAAAACCCCATCAACGCCAG
CGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGACGGCTGG
AAAATCTGATCGCCCAGCTGCCCGGCGAGAAGAAGAATGGCCTGTTCGGaAACCTG
ATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGAGCAACTTCGACCTGGCCGAG
GATGCCAAACTGCAGCTGAGCAAGGACACCTACGACGACGACCTGGACAACCTGCT
GGCCCAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCG
ACGCCATCCTGCTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCC
CTGAGCGCCTCTATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCT
GAAAGCTCTCGTGCGGCAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCGACC
AGAGCAAGAACGGCTACGCCGGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTTC
TACAAGTTCATCAAGCCCATCCTGGAAAAGATGGACGGCACCGAGGAACTGCTCGT
GAAGCTGAACAGAGAGGACCTGCTGCGGAAGCAGCGGACCTTCGACAACGGCAGC
ATCCCCCACCAGATCCACCTGGGAGAGCTGCACGCCATTCTGCGGCGGCAGGAAGA
TTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATCGAGAAGATCCTGACCTTCCG
CATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAGCAGATTCGCCTGGATGA
CCAGAAAGAGCGAGGAAACCATCACCCCCTGGAACTTCGAGGAAGTGGTGGACAA
GGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAGAACCTGC
CCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTACGAGTACTTCACCGTGTAT
AACGAGCTGACCAAAGTGAAATACGTGACCGAGGGAATGAGAAAGCCCGCCTTCCT
GAGCGGCGAGCAGAAAAAGGCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAA
GTGACCGTGAAGCAGCTGAAAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTC
CGTGGAAATCTCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCACG
ATCTGCTGAAAATTATCAAGGACAAGGACTTCCTGGACAATGAGGAAAACGAGGAC
ATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGA
GGAACGGCTGAAAACCTATGCCCACCTGTTCGACGACAAAGTGATGAAGCAGCTGA
AGCGGCGGAGATACACCGGCTGGGGCAGGCTGAGCCGGAAGCTGATCAACGGCATC
CGGGACAAGCAGTCCGGCAAGACAATCCTGGATTTCCTGAAGTCCGACGGCTTCGC
CAACAGAAACTTCATGCAGCTGATCCACGACGACAGCCTGACCTTTAAAGAGGACA
TCCAGAAAGCCCAGGTGTCCGGCCAGGGCGATAGCCTGCACGAGCACATTGCCAAT
CTGGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACAGTGAAGGTGGTGGA
CGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGAGAACATCGTGATCGAAATGG
CCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGAACAGCCGCGAGAGAATGAA
GCGGATCGAAGAGGGCATCAAAGAGCTGGGCAGCCAGATCCTGAAAGAACACCCC
GTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAATGG
GCGGGATATGTACGTGGACCAGGAACTGGACATCAACCGGCTGTCCGACTACGAT

FIG. 50B

GTGGACCATATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCGACAACAAGGTG
CTGACCAGAAGCGACAAGAACCGGGGCAAGAGCGACAACGTGCCCTCCGAAGAGG
TCGTGAAGAAGATGAAGAACTACTGGCGGCAGCTGCTGAACGCCAAGCTGATTACC
CAGAGAAAGTTCGACAATCTGACCAAGGCCGAGAGAGGCGGCCTGAGCGAACTGG
ATAAGGCCGGCTTCATCAAGAGACAGCTGGTGGAAACCCGGCAGATCACAAAGCAC
GTGGCACAGATCCTGGACTCCCGGATGAACACTAAGTACGACGAGAATGACAAGCT
GATCCGGGAAGTGAAAGTGATCACCCTGAAGTCCAAGCTGGTGTCCGATTTCCGGA
AGGATTTCCAGTTTTACAAAGTGCGCGAGATCAACAACTACCACCACGCCCACGAC
GCCTACCTGAACGCCGTCGTGGGAACCGCCCTGATCAAAAAGTACCCTAAGCTGGA
AAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGCGGAAGATGATCGCCA
AGAGCGAGCAGGAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGCAACATC
ATGAACTTTTTCAAGACCGAGATTACCCTGGCCAACGGCGAGATCCGGAAGCGGCC
TCTGATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGATT
TTGCCACCGTGCGGAAAGTGCTGAGCATGCCCCAAGTGAATATCGTGAAAAAGACC
GAGGTGCAGACAGGCGGCTTCAGCAAAGAGTCTATCCTGCCCAAGAGGAACAGCGA
TAAGCTGATCGCCAGAAAGAAGGACTGGGACCCTAAGAAGTACGGCGGCTTCGACA
GCCCCACCGTGGCCTATTCTGTGCTGGTGGTGGCCAAAGTGGAAAAGGGCAAGTCC
AAGAAACTGAAGAGTGTGAAAGAGCTGCTGGGGATCACCATCATGGAAAGAAGCA
GCTTCGAGAAGAATCCCATCGACTTTCTGGAAGCCAAGGGCTACAAAGAAGTGAAA
AAGGACCTGATCATCAAGCTGCCTAAGTACTCCCTGTTCGAGCTGGAAAACGGCCG
GAAGAGAATGCTGGCCTCTGCCGGCGAACTGCAGAAGGGAAACGAACTGGCCCTGC
CCTCCAAATATGTGAACTTCCTGTACCTGGCCAGCCACTATGAGAAGCTGAAGGGCT
CCCCCGAGGATAATGAGCAGAAACAGCTGTTTGTGGAACAGCACAAGCACTACCTG
GACGAGATCATCGAGCAGATCAGCGAGTTCTCCAAGAGAGTGATCCTGGCCGACGC
TAATCTGGACAAAGTGCTGTCCGCCTACAACAAGCACCGGGATAAGCCCATCAGAG
AGCAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCCCCTGCCG
CCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACCAAAGAG
GTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGAT
CGACCTGTCTCAGCTGGGAGGCGACAAAAGGCCGGCGGCCACGAAAAAGGCCGGC
CAGGCAAAAAAGAAAAAG

P2A-EGFP

ggaagcggagccactaacttctccctgttgaaacaagcaggggatgtcgaagaggatcccgggccaGTGAGCAAGGGCGA
GGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACG
GCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTG
ACCCTGAAGTTCATCT

FIG. 50C

GCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACG
GCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGT
CCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGC
AACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCAT
CGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGG
AGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGC
ATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGC
CGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACA
ACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGAT
CACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAG
CTGTACAAG

WPRE
Cgataatcaacctctggattacaaaatttgtgaaagattgactggtattcttaactatgttgctccttttacgctatgtggatacgctgctttaatgc
ctttgtatcatgctattgcttcccgtatggctttcattttctcctccttgtataaatcctggttgctgtctctttatgaggagttgtggcccgttgtcagg
caacgtggcgtggtgtgcactgtgtttgctgacgcaacccccactggttggggcattgccaccacctgtcagctcctttccgggactttcgctt
tccccctccctattgccacggcggaactcatcgccgcctgccttgcccgctgctggacaggggctcggctgttgggcactgacaattccgtg
gtgttgtcggggaaatcatcgtcctttccttggctgctcgcctgtgttgccacctggattctgcgcgggacgtccttctgctacgtcccttcggc
cctcaatccagcggaccttccttcccgcggcctgctgccggctctgcggcctcttccgcgtcttcgccttcgccctcagacgagtcggatctc
cctttgggccgcctccccgcatcg

bGHpolyA
cgacCTCGACtgtgccttctagttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctggaaggtgccactcccactgt
ccttcctaataaaatgaggaaattgcatcgcattgtctgagtaggtgtcattctattctgggggggtggggtggggcaggacagcaagggggg
aggattgggaagacaatGgcaggcatg

loxP-SV40polyAx3-loxP
ataacttcgtataatgtatgctatacgaagttattcgcgatgaataaatgaaagcttgcagatctgcgactctagaggatctgcgactctagagg
atcataatcagccntaccacattttgtagaggtttactngctttaaaaaacctcccacacctcccccgaaccgaaacataaaatgaatgcaa
ttgttgttgttaacttgtttattgcagcttataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttcactgcattctagt
tgtggtttgtccaaactcatcaatgtatcttatcatgtctggatctgcgactctagaggatcataatcagccataccacatttgtagaggttttactt
gctttaaaaaacctcccacacctcccccgaaccgaaacataaaatgaatgcaattgttgttgttaacttgtttattgcagcttataatggttaca
aataaagcaatagcatcacaaatttcacaaataaagcatttttttcactgcattctagttgtggtttgtccaaactcatcaatgtatcttatcatgtct
ggatctgcgactctagaggatcataatcagccataccacatttgtagaggtttactgctttaaaaaacctcccacacctcccccgaaccgga
aacataaaatgaatgcaattgttgttgttaacttgtttattgcagcttataatggttacaaata

FIG. 50D

aagcaatagcatcacaaatttcacaaataaagcatttttttcactgcattctagttgtggtttgtccaaactcatcaatgtatcttatcatgtctggat
ccccatcaagctgatccggaacccttaatataacttcgtataatgtatgctatacgaagttat

Rosa26 short homology arm
caggccctccgagcgtggtggagccgttctgtgagacagccgggtacgagtcgtgacgctggaaggggcaagcgggtggtgggcagg
aatgcggtccgcccctgcagcaaccggaggggggagggagaagggagcggaaaagtctccaccggacgcggccatggctcgggggggg
gggggcagcggaggaGcgcttccggccgacgtctcgtcgctgattggcttCttttcctcccgccgtgtgtgaaaacacaaatggcgtgtt
ttggttggcgtaaggcgcctgtcagttaacggcagccggagtgcgcagccgccggcagcctcgctctgcccactgggtggggcgggag
gtaggtgggggtgaggcgagctgGacgtgcgggcgcggtcggcctctggcgggggcgggggaggggaggggaggggtcagcgaaagta
gctcgcgcgcgagcggccgcccaccctccccttcctctgggggagtcgttttacccgccgccggccgggcctcgtcgtctgattggctctc
ggggcccagaaaactggcccttgccattggctcgtgttcgtgcaagttgagtccatccgccggccagcgggggcggcgaggaggcgctc
ccaggttcggccctcccctcggcccccgcgccgcagagtctggccgcgcgccccctgcgcaacgtggcaggaagcgcgcgctgggggc
ggggacgggcagtagggctgagcggctgcgggggcgggtgcaagcacgtttccgacttgagttgcctcaagagggcgtgctgagccag
acctccatcgcgcactccgggggagtggagggaaggagcgagggctcagttgggctgttttggaggcaggaagcacttgctctccccaaagt
cgctctgagttgttatcagtaagggagctgcagtggagtaggcgggggagaaggccgcaccctctccggagggggggaggggagtgttgc
aatacctttctgggagttctctgctgcctcctggcttctgaggaccgccctgggcctgggagaatccctttcccccctttccctcgtgatctgcaa
ctccagtctttctag

Rosa26 long homology arm
agatgggcgggagtcttctgggcaggcttaaaggctaacctggtgtgtgggcgttgtcctgcaggggaattgaacaggtgtaaaattggag
ggacaagacttcccacagattttcggttttgtcgggaagttttttaataggggcaaataaggaaaatgggaggataggtagtcatctgggggtttt
atgcagcaaaactacaggttattattgcttgtgatccgcctcggagtatttccatcgaggtagattaaagacatgctcaccgagtttatactct
cctgcttGAGATCCTTACTACAGTATGAAATTACAGTGTCGCGAGTTAGACTATGTAAGC
AGAATTTTAATCATtttaaagagcccagtacttcatatccatttctcccgctccttctgcagccttatcaaaaggtaTttagaaca
ctcatttagccccatttttcatttattatactggcttatccaaccctagacagagcattggcatttttccctttcctgatcttagaagtctgatgactca
tgaaaccagacagattagttacatacaccacaaatcgaggctgtagctggggcctcaacactgcagttcttttataactccttagtacacttttg
ttgatcctttgccttgatccttaatttcagtgtctatcacctctcccgtcaggtggtgttccacatttgggcctattctcagtccagggagttttaca
acaatagatgtattgagaatccaacctaaagcttaactttccactcccatgaatgcctctctcctttttctccattTATAAACTGAGCT
ATTAACCATTAATGGTTTCCAGGTGGATGTCTCCTCCCCCAATATTACCTGATGTATC
TTACATATTGCCAGGCTGATATTTTAAGACATTAAAAGGTATATTTCATTATTGAGCC
ACATGGTATTGATTACTGCTtactaaaatttttgtcattgtacacatctgtaaaaggtggttccttttggaatgcaaagttcaggt
gtttgttgtcttttcctgacctaaggtcttgtgagcttgtatttttttctatttaagcagtgcttttctcttggactggcttgactcatggcattctacacgtta
ttgctggtctaaatgtgattttgccaagcttcttcaggacctataatttttgcttgacttgtagccaaacacaagtaaaatgattaagcaacaaatgt
atttgtgaagcttggttttaggttgttgtgttgtgtgcttgtgctctataataatactatccaggggctggagaggtggctcggagttcaagag
cacagactgctcttccagaagtc

FIG. 50E

ctgagttcaatcccagcaaccacatggtggctcacaaccatctgtaatgggatctgatgccctcttctggtgtgtctgaagaccacaagtgta
ttcacattaaaataaataaaTCCTCCTTCTTCTTCTTTTTTTTTTTTTtAAAGAGAATACTGTCTCCAG
TAGAAtTTACTGAAGTAATGAAATACTTTGTGTTTGTTCCAATATGGTAGCCAATAAT
CAAATtACTCTTTaAGCACTGGAAATGTtACCAAGGAACTAaTTTTtATTTgAAGTGTaA
CTGTGGACAGAGGAGCCATAACTGCAGACTTGTGGGATACAGAAGACCAATGCAGA
CtTTAATGTCTTTTCTCTTACACTAAGCAATAAAGAAATAAAAATTGAACTTCTAGTA
TCCTATTTGTTtAAACTGCTAGCTTTACtTAACTTTTGTGCTTCATCTATACAAAGCTG
AAAGCTAAGTCTGCAGCCATTACTAAACATGAAAGCAAGTAATGATAATTTTGGATT
TCAAAAATGTAGGGCCAGAGTTTAGCCAGCCAGTGGTGGTGCTTGCCTTTATGCCtTT
AATCCCAGCACTCTGGAGGCAGAGACAGGCAGATCTCTGAGTTTGAGCCCAGCCTG
GtCTACACATCAAGTTCTATCTAGGATAGCCAGGAATACACACAGAAACCCTGTTGG
GGAGGGGGGCTCTGAGATTTCATAAAATTATAATTGAAGCATTCCCTAATGAGCCAC
TATGGATGTGGCTAAATCCGTCTACCTTTCTGATGAGATTTGGGTATTATTTTTTCTG
TCTCTGCTGTTGGTTGGGTCTTTTGACACTGTGGGCTTTCTTtAAAGCCTCCTTCCTGC
CATGTGGTCTCTTGTTTGCTACTAACTTCCCATGGCTTAAATGGCATGGCTTTTTGCC
TTCTAAGGGCAGCTGCTGAGATTTGCAGCCTGATTTCCAGGGTGGGGTTGGGAAATC
TTTCAAACACTAAAATTGTCCTTTAAtTTTTTTTTTAAAAAATGGGTTATATAATAAA
CCTCATAAAATAGTTATGAGGAGTGAGGTGGACTAATATTAAaTGAGTCCCTCCCCT
ATAAAAGAGCTATTAAGGCTTTTTGTCTTATACTtAACTTTTTTTTTAAATGTGGTATC
TTTAGAACCAAGGGTCTTAGAGTTTTAGTATACAGAAACTGTTGCATCGCTTAATCA
GATTTTCTAGTTTCAAATCCAGAGAATCCAAATTCTTCACAGCCAAAGTCAAATTAA
GAATTTCTGACTTTtAATGTTAaTTTGCtTACTGTGAATATaAAAATGATAGCTTTTCCT
GAGGCAGGGTCTCACTATGTATCTCTGCCTGATCTGCAACAAGATATGTAGACTAAA
GTTCTGCCTGCTTTTGTCTCCTGAATACTAAGGTTAAAATGTAGTAATACTTTTGGAA
CTTGCAGGTCAGATTCTTTTATAGGGGACACACTAAGGGAGCTTGGGTGATAGTTGG
TAAAtgtgtttaagtgatgaaaacttgaattattatcaccgcaacctacttttttaaaaaaaaaagccaggcctgttagagcatgctTaaggg
atcctaggacttgctgagcacacaAGAGTAGtTACTTGGCAGGCTCCTGGTGAGAGCATATTTCAA
AAAACAAGGCAGACAACCAAGAAACTACAGTtAAGGTTACCTGTCTTTaAACCATCT
GCATATACACAGGGATATTAAAATATTCCAAATAATATTTCATTCAAGTTTTCCCCC
ATCAAATTGGGACATGGATTTCTCCGGTGAATAGGCAGAGTTGGAAACTAAACAAA
TGTTGGTTTTGTGATTTGTGAAATTGTTTTCAAGTGATAGTTAAAGCCCATGAGATAC
AGAACAAAGCTGCTATTTCGAGGTCTCTTGGTTtATACTCAGAAGCACTTCTTTGGGT
TTCCCTGCACTATCCTGATCATGTGCTAGGCCTACCTTAGGCTGATTGTTGTTCAAAT
aAACTTAAGTTTCCTGTCAGGTGATGTCATATGATTTCATATATCAAGGCAAAACATG
TTATATATGTTAAACATTTGTACTTAATGTGAAAGTTAGGTCTTTGTGGGTT

FIG. 50F

TGATTTTtAAtTTTCAAAACCTGAGCTAAATAAGTCATTTTtACATGTCTTACATTTGGT
GgAATTGTATaATTGTGGTTTGCAGGCAAGACTCTCTGACCTAGTAACCCTaCCTATA
GAGCACTTTGCTGGGTCACAAGTCTAGGAGTCAAGCATTTCACCTTGAAGTTGAGAC
GTTTTGTTAGTGTATACTAGTTtATATGTTGGAGGACATGTTTATCCAGAAGATATTC
AGGACTATTTTTGACTGGGCTAAGGAATTGATTCTGATTAGCACTGTTAGTGAGCAT
TGAGTGGCCTTTAGGCTTGAATTggagtcacttgtatatctcaaataatgctggcctttttaaaagccctgttctttatca
ccctgttttctacataatttttgttcaaagaaatacttgtttggaTCTCCTTTTGACAACAATAGCATGTTTTCAAG
CCATATTTTTTTTCCTTTTTTTTTTTTTTTTTGGTTTTTCGAGACAGGGTTTCTCTGTAT
AGCCCTGGCTGTCCTGGAACTCACTTTGTAGACCAGGCTGGCCTCGAACTCAGAAAT
CCGCCTGCCTCTGCCTCCTGAGTGCCGGGATTAAAGGCGTGCACCACCACGCCTGGC
TAAGTTGGATATTTTGTtATATAACTATAACCAATACTAACTCCACTGGGTGGATTTT
TAATTCAGTCAGTAGTCTTAAGTGGTCTTTATTGGCCCTTcATTAAAATCTACTGTTC
ACTCTAACAGAGGCTGTTGGtACTAGTGGCACtTAAGCAACTTCCTACGGATATACTA
GCAGAtTAAGGGTCAGGGATAGAAACTAGTCTAGCGTTTTGTATACCTACCAGCTTtA
TACTACCTTGTTCTGATAGAAATATTTcAGGACATCTAGCTT

pPGK-Neo-pPGK-polyA
aattctaccgggtaggggaggcgcttttcccaaggcagtctggagcatgcgctttagcagccccgctgggcacttggcgctacacaagtgg
cctctggcctcgcacacattccacatccaccggtaggcgccaaccggctccgttcttttggtggccccttcgcgccaccttctactcctcccct
agtcaggaagttcccccccgccccgcagctcgcgtcgtgcaggacgtgacaaatggaagtagcacgtctcactagtctcgtgcagatgga
cagcaccgctgagcaatggaagcgggtaggcctttggggcagcggccaatagcagctttgctccttcgctttctgggctcagaggctggg
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acgcttcaaaagcgcacgtctgccgcgctgttctcctcttcctcatctccgggcctttcgacctgcaatgccgctagcgaagttcctattctct
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accgacctgtccggtgccctgaatgaactgcaggacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgt
gctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcaggatctcctgtcatctcaccttgctcctgcc
gagaaagtatccatcatggctgatgcaatgcggcggctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgca
tcgagcgagcacgtactcggatggaagccggtcttgtcgatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactg
ttcgccaggctcaaggcgcgcatgcccgacggcgatgatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatg
gccgcttttctggattcatcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgctgaagag
cttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatcgccttcttgacgagt
tcttctgaggggatccgctgtaagtctgcagaaattgatgatctattaaacaataaagatgtccactaaaatggaagttttcctgtcatacttttgtt
aagaagggtgagaacagagtacctacattttgaatggaaggattggagctacgggggtgggggtggggtgggattagataaatgcctgct
ctttactgaaggctctttactattgctttatgataatgtttcatagttg

FIG. 50G

gatatcataatttaaacaagcaaaaccaaattaagggccagctcattcctcccactcatgatctatagatctatagatctctcgtgggatcattgt
ttttctcttgattcccactttgtggttctaagtactgtggtttccaaatgtgtcagtttcatagcctgaagaacgagatcagcagcctctgttccaca
tacacttcattctcagtattgttttgccaagttctaattccatcagaaagc

pPGK-DTA
TACCGGGTAGGGGAGGCGCTTTTCCcAAGGCAGTCTGgAGCATGCGCtTTAGCAGCCC
CGCTgGGCACTTGGCGCTACACAAGTGGCCTCTGGCCTCGCACACATTCCACATCCA
CCGGTAGGCGCCAACCGGCTCCGTTCTTTGGTGGCCCCTTCGCGCCACCTTCTACTCC
TCCCCTAGTCAGGAAGTTCCCCCCCGCCCCGCAGCTCGCGTCGTGcAGGACGTGACA
AATGGAAGTAGCACGTCTCACTAGTCTCGTgCAGATGGACAGCACCGCTGAGCAATG
GAAGCGGGTAGGCCTTTGGGGCAGCGGCCAATAGCAGCTTTGCTCCTTCGCTTTCTG
GGCTCAGAGGCTGGGAAGGGGTGGGTCCGGGGGCGGGCTCAGGGGCGGGCTCAGG
GGCGGGGCGGGCGCCCGAAGGTCCTCCGGAGGCCCGGCATTCTGCACGCTTCAAAA
GCGCACGTCTGCCGCGCTGTTCTCCTCTTCCTCATCTCCGGGCCTTTCGACCTGCAGG
TCCTCGCCATggatcctgatgatgttgttGattcttctaaAtcttttgtGatggaaaactttcttcgtaccacgggactaaacctggtt
atgtagattccattcaaaaaggtatacaaaagccaaaatctggtacacaaggaaattatgacgatgattggaaagggttttatagtaccgacaa
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acggaagagtttatcaaaaggttcggtgatggtgcttcgcgtgtagtgctcagccttcccttcgctgaggggagttctagcgttgaatatattaa
taactgggaacaggcgaaagcgttaagcgtagaacttgagattaatttgaaacccgtggaaaacgtggccaagatgcgatgtatgagtata
tggctcaagcctgtgcaggaaatcgtgtcaggcgatctcttgtgaaggaaccttacttctgtggtgtgacataattggacaaactacctacag
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FIG. 50H

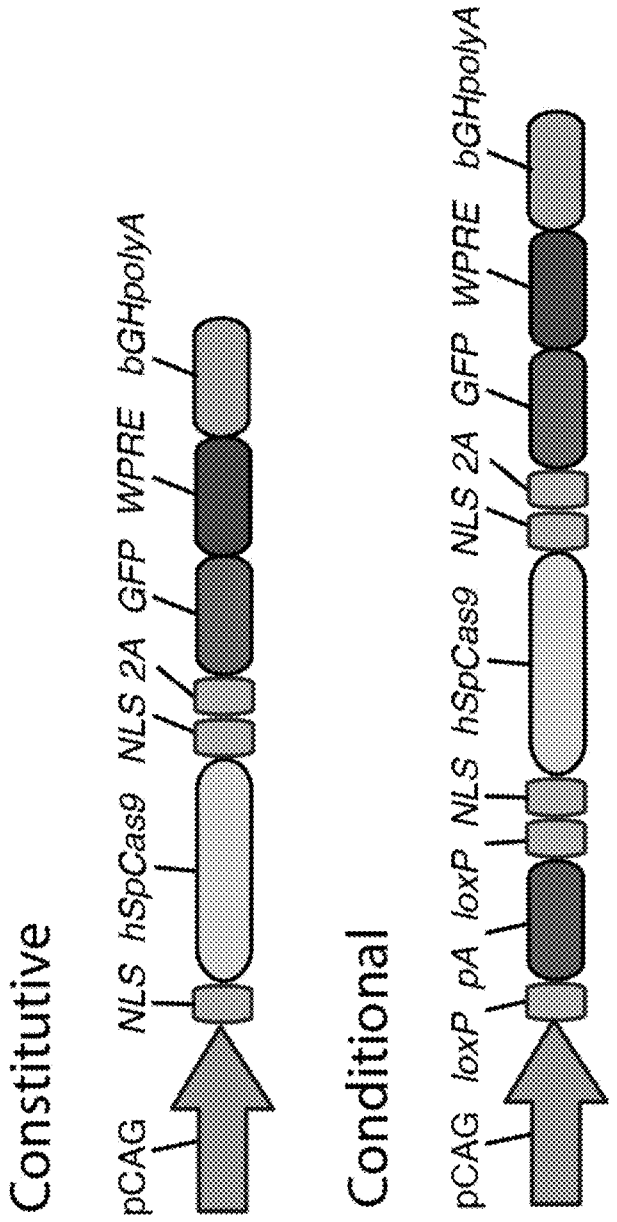

Constitutive
pCAG
NLS
hSpCas9
NLS
2A
GFP
WPRE
bGHpolyA
Conditional
pCAG
loxP
pA
loxP
NLS
hSpCas9
NLS
2A
GFP
WPRE
bGHpolyA

FIG. 51

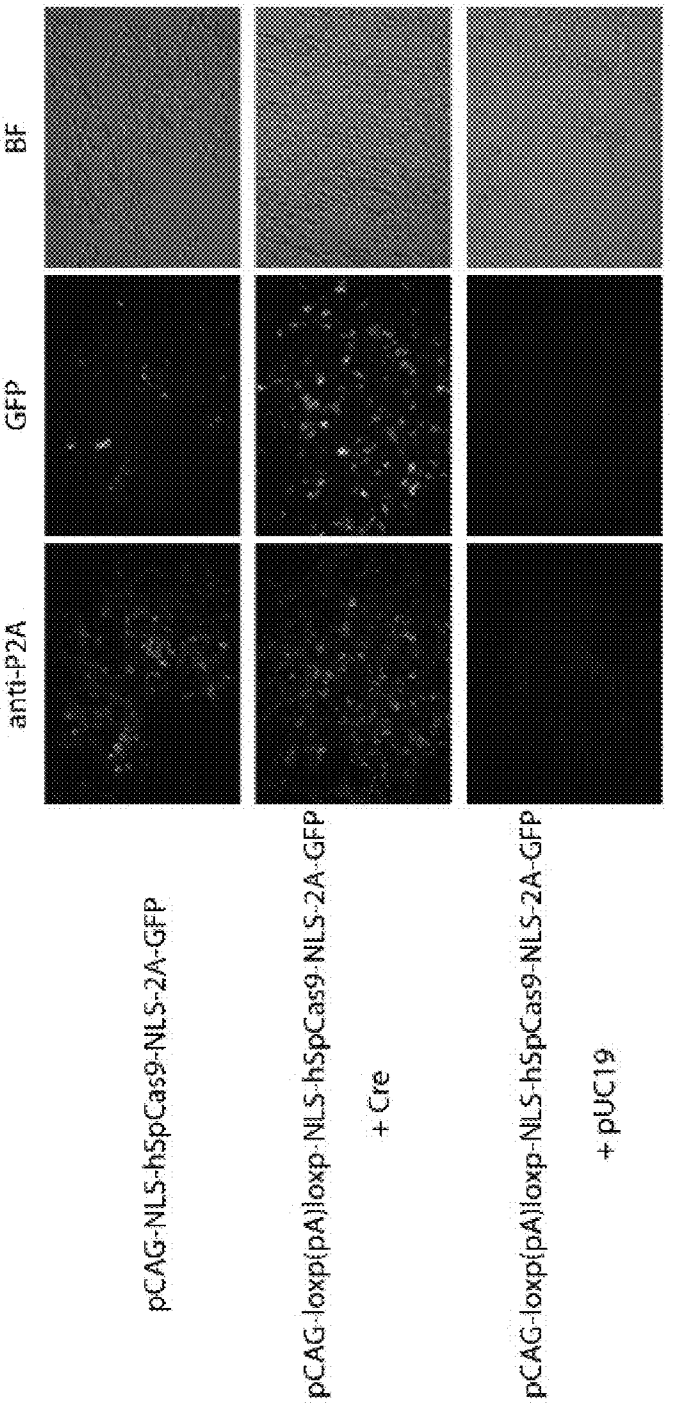

anti-P2A
GFP
BF
pCAG-NLS-hSpCas9-NLS-2A-GFP
pCAG-loxp(pA)loxp-NLS-hSpCas9-NLS-2A-GFP
+ Cre
pCAG-loxp(pA)loxp-NLS-hSpCas9-NLS-2A-GFP
+ pUC19

FIG. 52

Validation of Cas9 nuclease activity by Surveyor

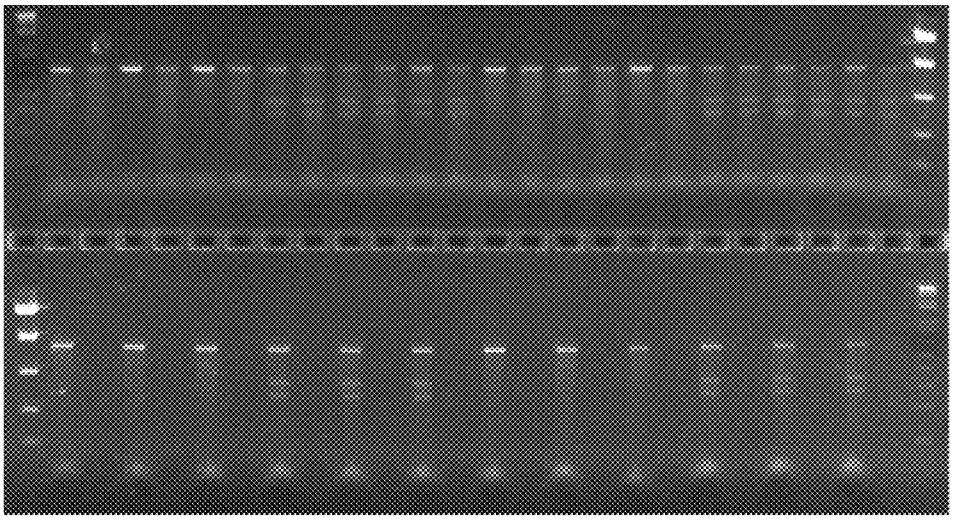


FIG. 53

		Average	StDev
pCAG-loxp(pA)loxp-NLS-hSpCas9-NLS-2A-GFP	Clone 1	32.1	7.1
	Clone 2	27.3	3.5
	Clone 3	35.9	1.4
	Clone 4	39.0	4.7
pCAG-NLS-hSpCas9-NLS-2A-GFP	Clone 1	26.9	1.3
	Clone 2	33.1	2.7

FIG. 54

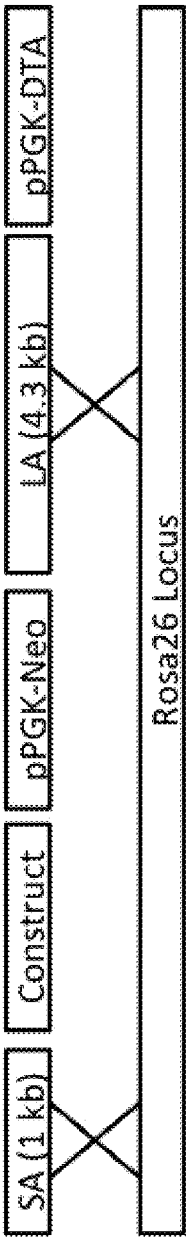


FIG. 55

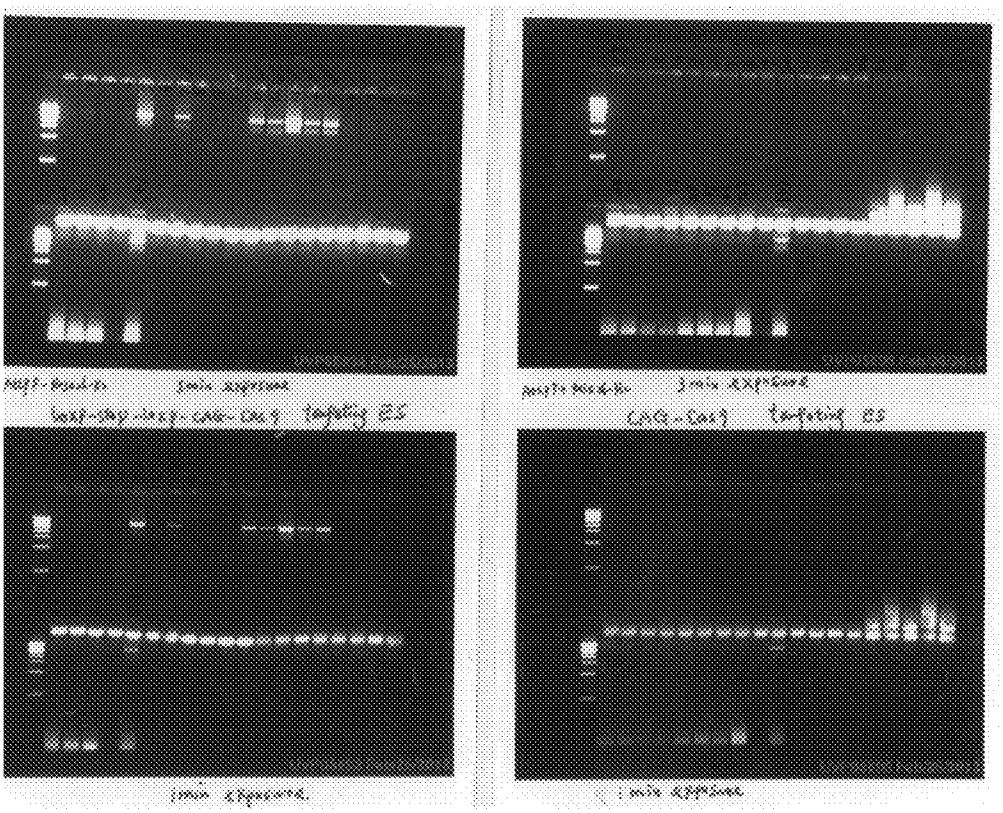


FIG. 56

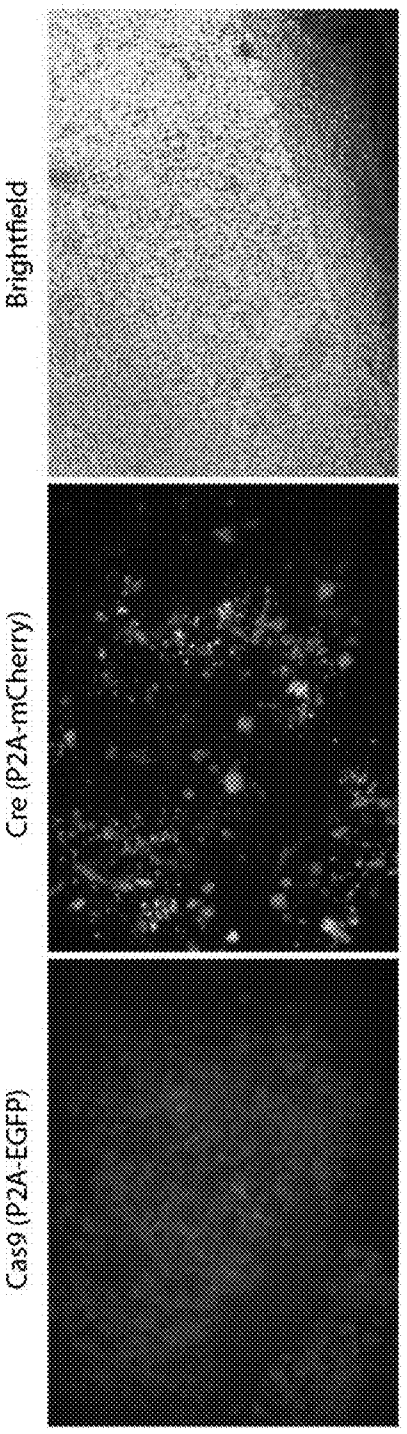


FIG. 57

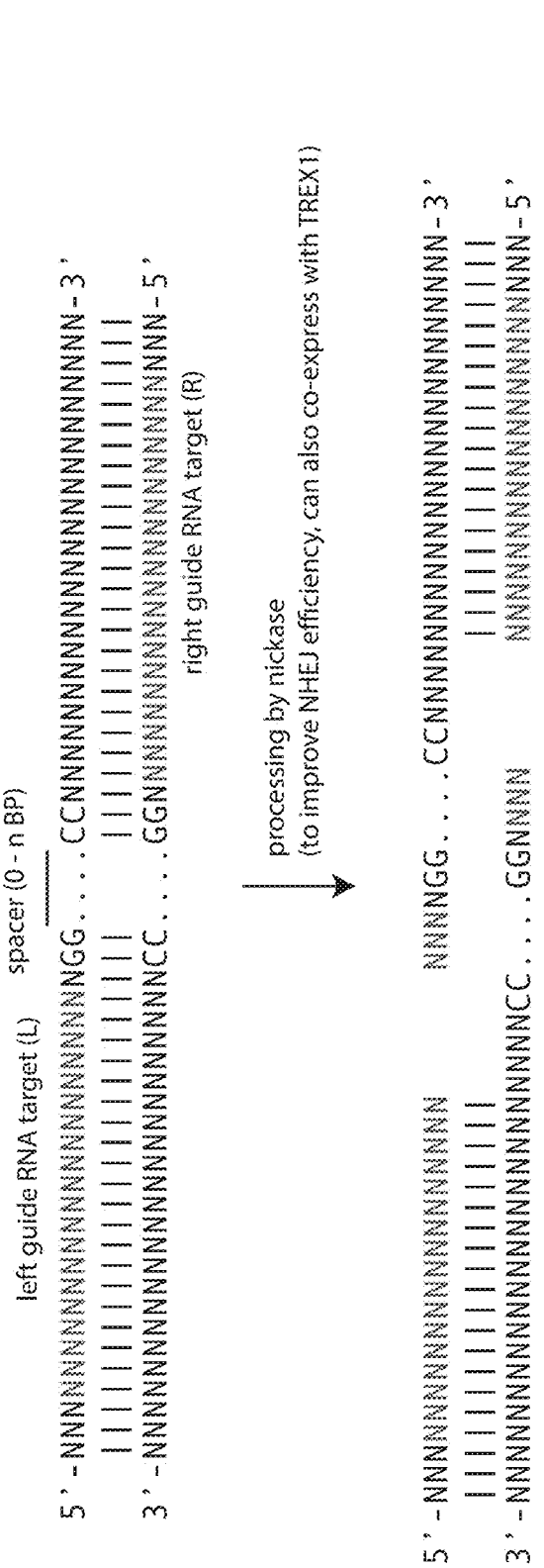

left guide RNA target (L)
spacer (0 - n BP)
5'-NNNNNNNNNNNNNNNNNNNNGG. . . . CCNNNNNNNNNNNNNNNNNNNN-3'
3'-NNNNNNNNNNNNNNNNNNNNCC. . . . GGNNNNNNNNNNNNNNNNNNNN-5'
right guide RNA target (R)
processing by nickase
(to improve NHEJ efficiency, can also co-express with TREX1)
5'-NNNNNNNNNNNNNNNNNNNN NNNNGG. . . . CCNNNNNNNNNNNNNNNNNNNN-3'
3'-NNNNNNNNNNNNNNNNNNNNCC. . . . GGNNNN NNNNNNNNNNNNNNNNNNNN-5'

FIG. 58

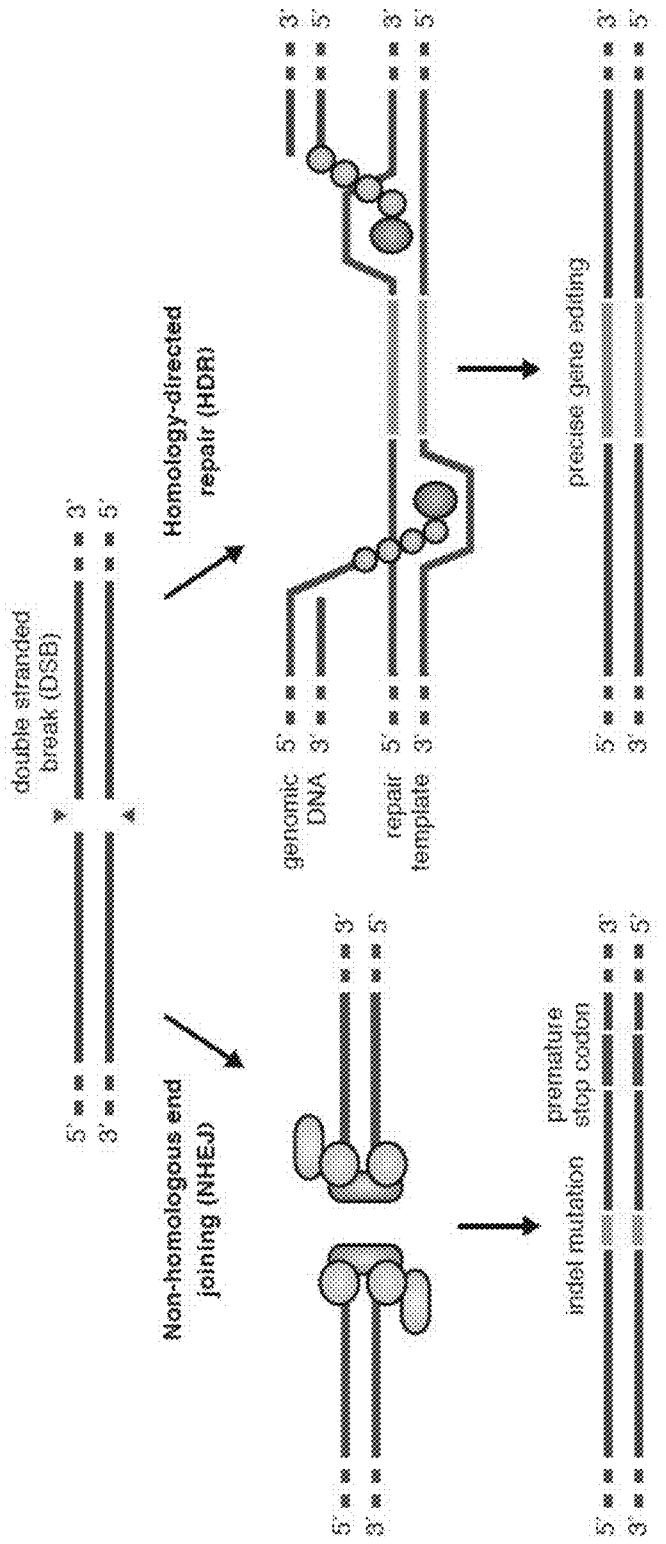

double stranded break (DSB)
5'
3'
3'
5'
Homology-directed repair (HDR)
genomic DNA
repair template
precise gene editing
Non-homologous end joining (NHEJ)
indel mutation
premature stop codon

FIG. 59

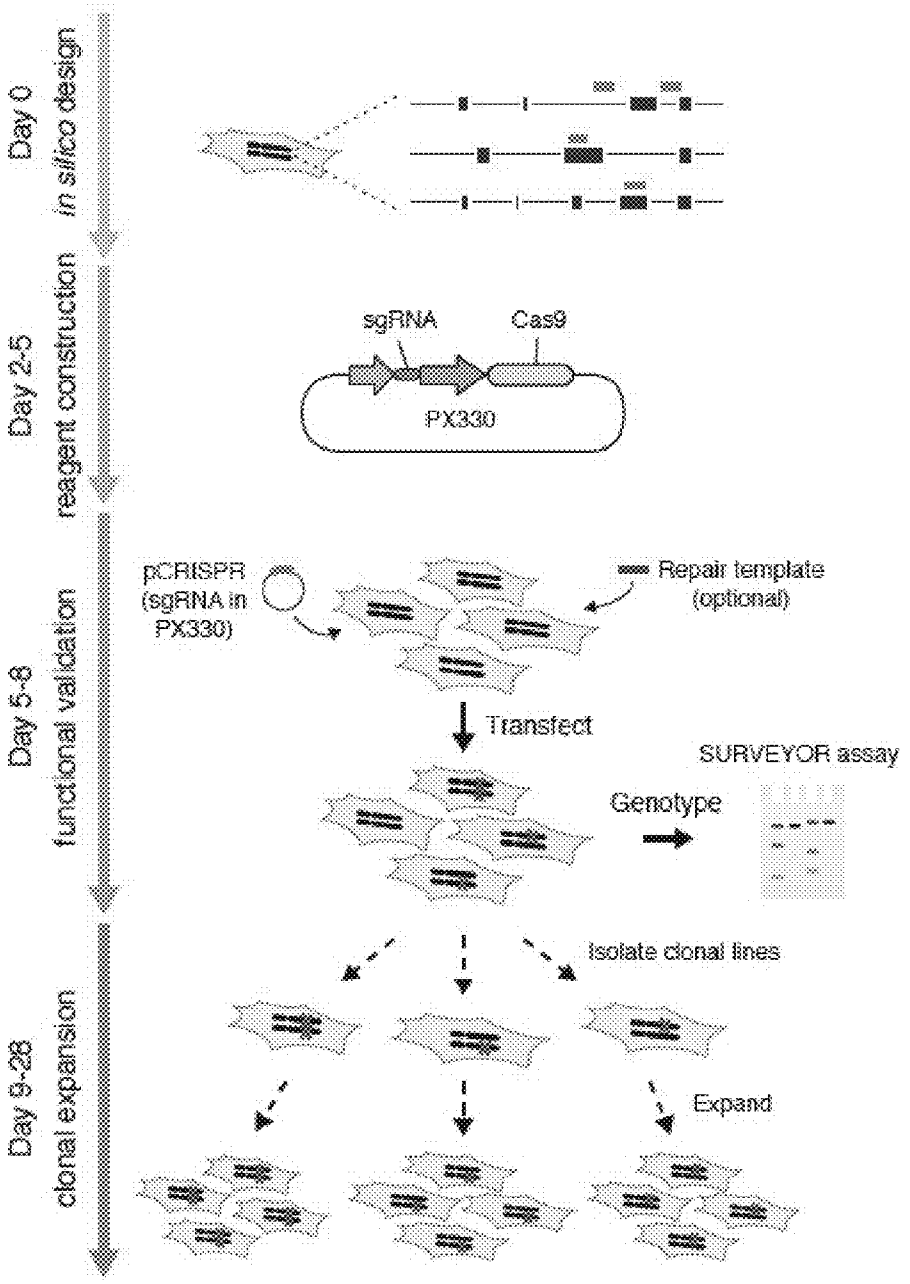

Day 0
in silico design
Day 2-5
reagent construction
sgRNA
Cas9
PX330
Day 5-8
functional validation
pCRISPR
(sgRNA in
PX330)
Repair template
(optional)
Transfect
SURVEYOR assay
Genotype
Day 9-28
clonal expansion
Isolate clonal lines
Expand

FIG. 60

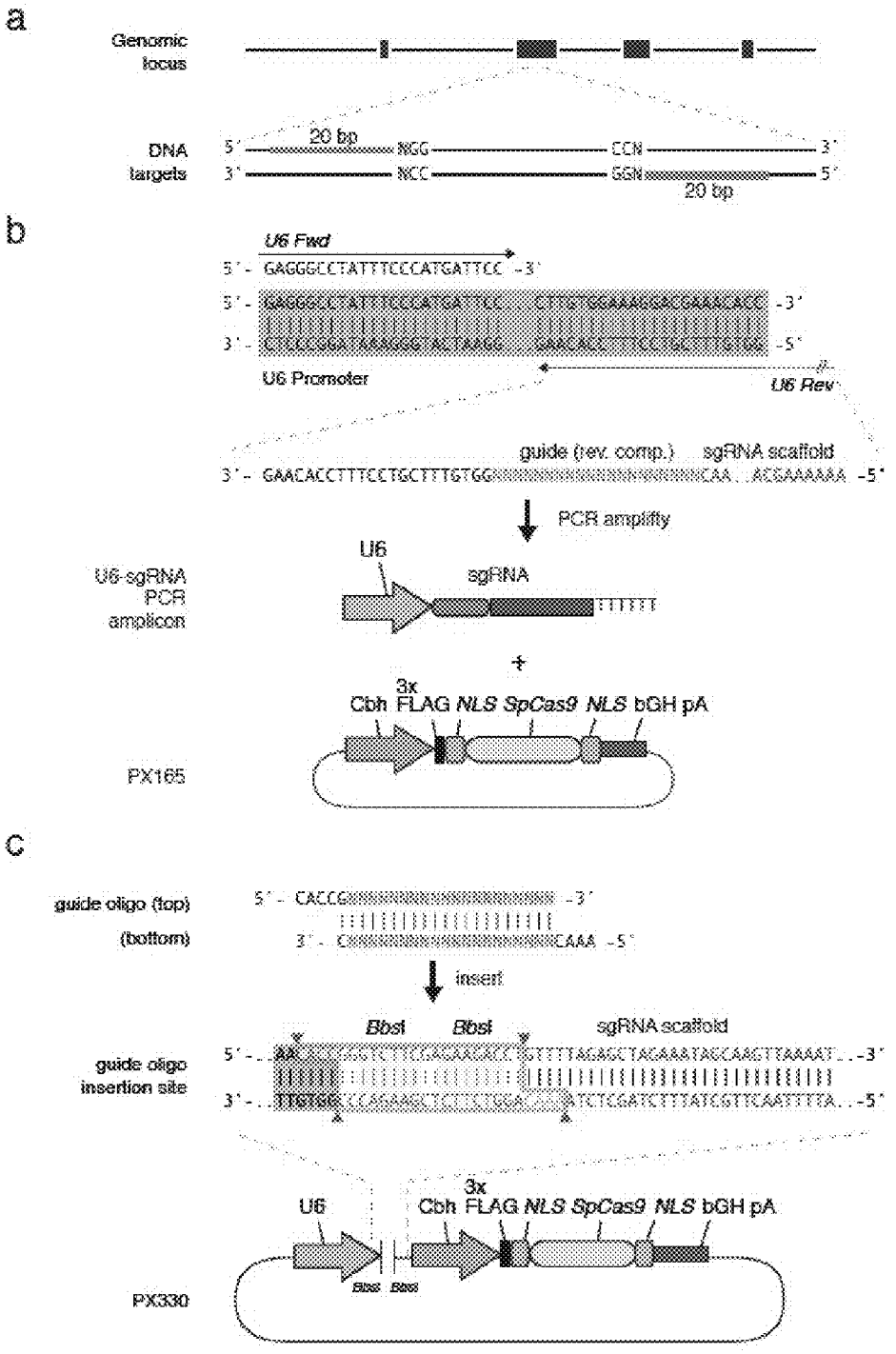
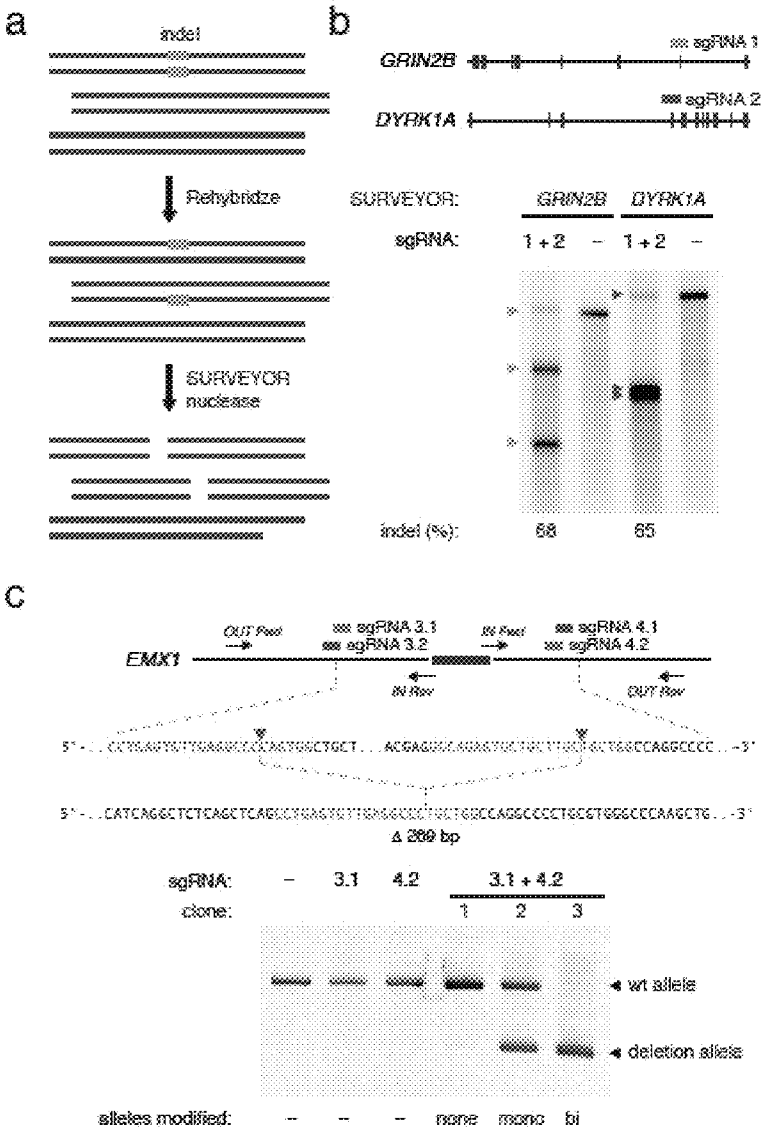

a
Genomic locus
DNA targets
20 bp
NGG
CCN
NCC
GGN
20 bp
b
U6 Fwd
U6 Promoter
U6 Rev
guide (rev. comp.)
sgRNA scaffold
PCR amplify
U6
sgRNA
U6-sgRNA PCR amplicon
3x Cbh FLAG NLS SpCas9 NLS bGH pA
PX165
c
guide oligo (top)
(bottom)
insert
BbsI
BbsI
sgRNA scaffold
guide oligo insertion site
U6
3x Cbh FLAG NLS SpCas9 NLS bGH pA
PX330

FIG. 61



d

Approx. deletion		Number of clones				
sgRNAs	size (bp)	+/+	Δ/+	Δ/Δ	inversion	total
3.1 + 4.1	282	12	10	1	0	23
3.2 + 4.1	237	20	16	2	0	38
3.1 + 4.2	425	4	14	5	0	23
3.2 + 4.2	259	6	11	4	0	21

FIG. 62

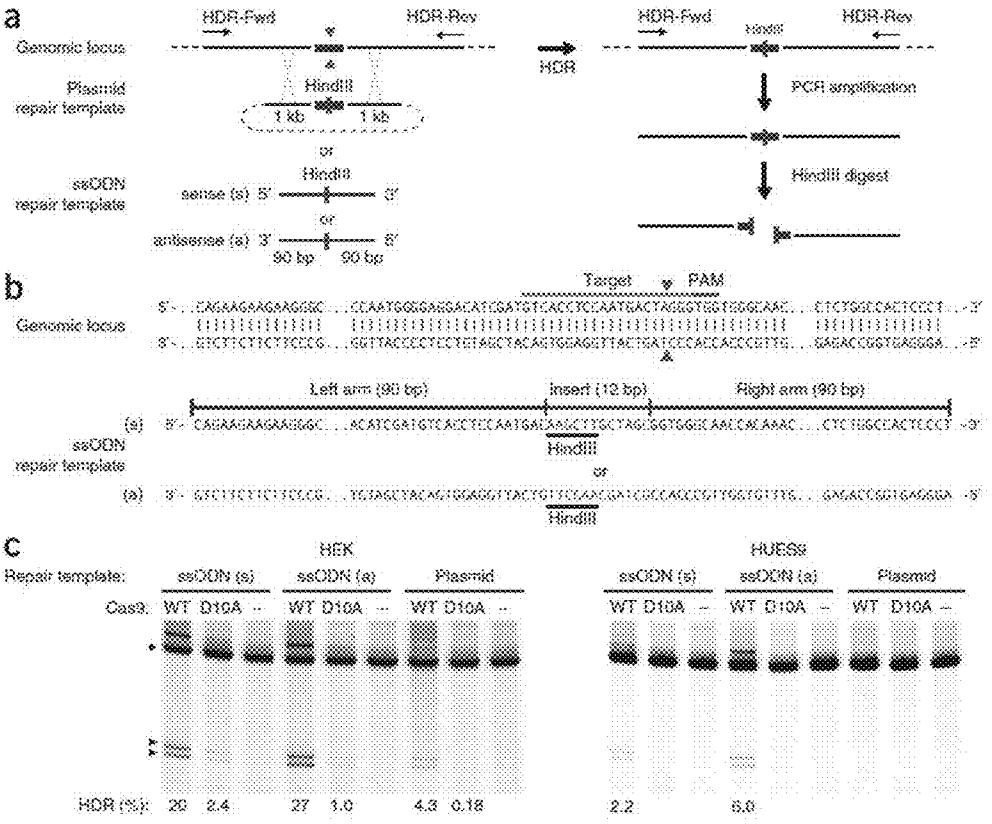

a
HDR-Fwd
HDR-Rev
Genomic locus
Plasmid repair template
HindIII
1 kb
1 kb
or
ssODN repair template
sense (s) 5'
3'
or
antisense (a) 3'
5'
90 bp
90 bp
HDR
HDR-Fwd
HindIII
HDR-Rev
PCR amplification
HindIII digest
b
Target
PAM
Genomic locus
Left arm (90 bp)
Insert (12 bp)
Right arm (90 bp)
(s)
ssODN repair template
HindIII
or
(a)
HindIII
c
HEK
HUES9
Repair template:
ssODN (s)
ssODN (a)
Plasmid
Cas9:
WT D10A –
HDR (%):
20 2.4
27 1.0
4.3 0.18
2.2
6.0

FIG. 63A-C

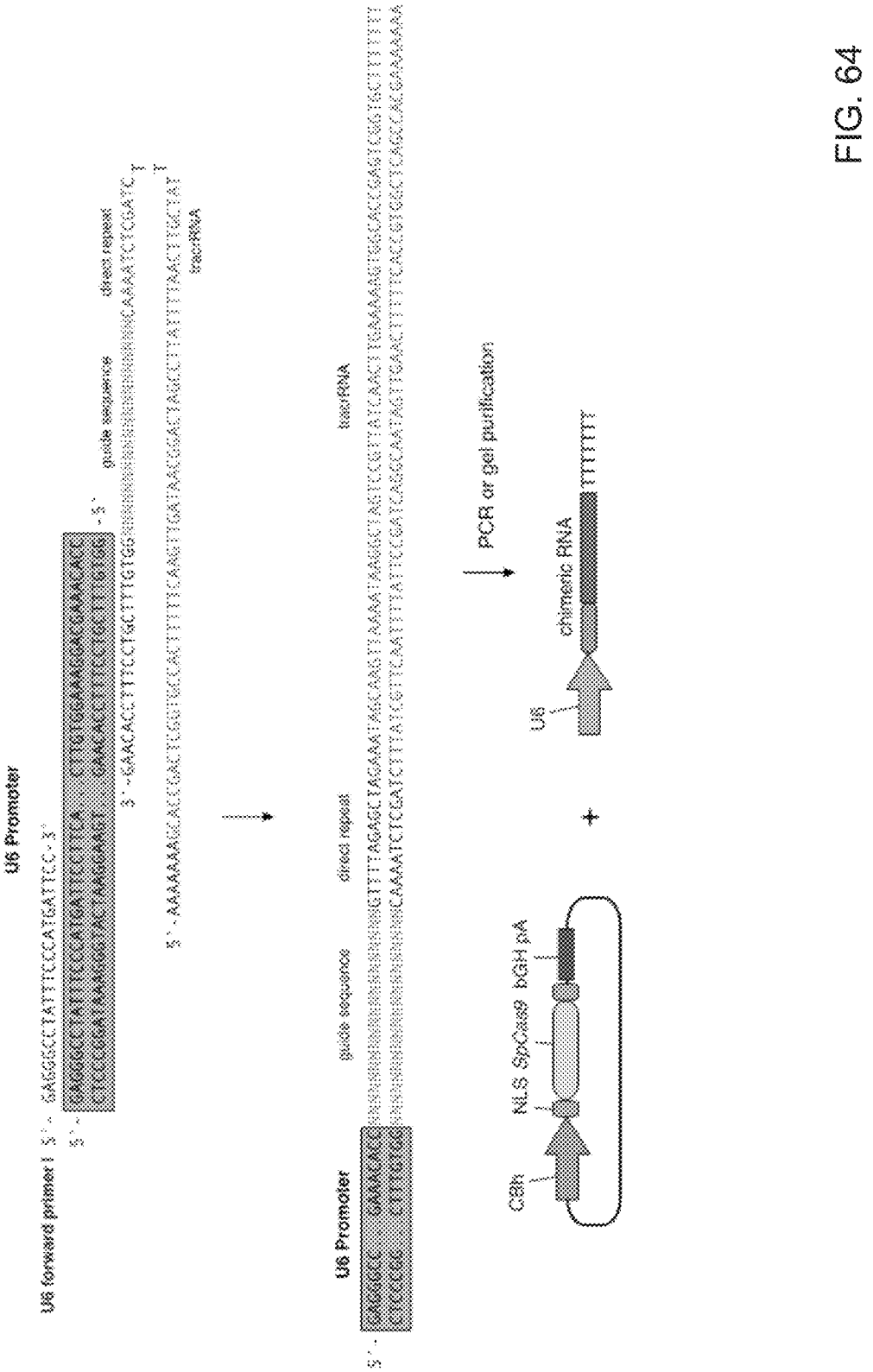
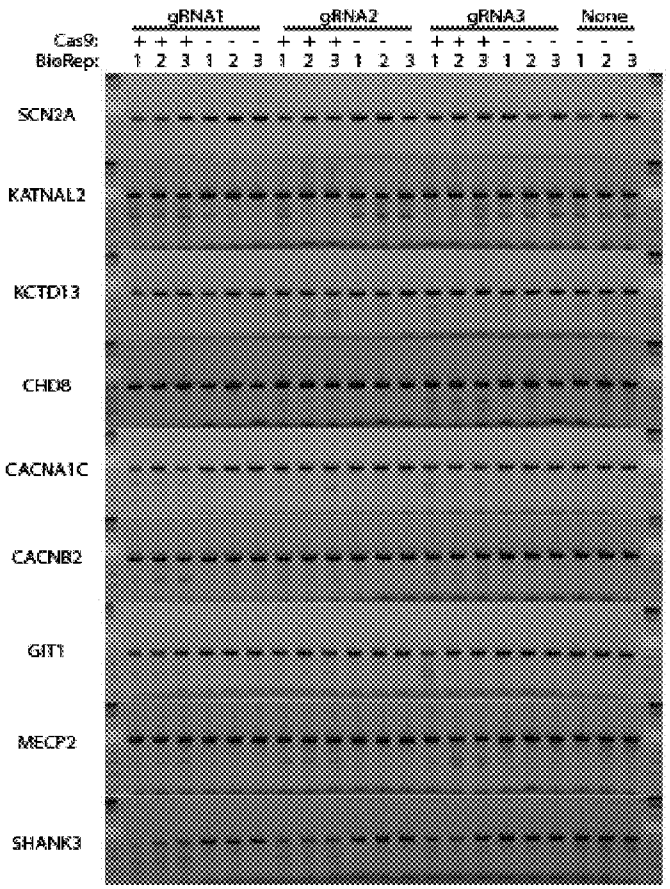

U6 Promoter
U6 forward primer
guide sequence
direct repeat
tracrRNA
PCR or gel purification
U6
chimeric RNA
TTTTTTT
CBh
NLS SpCas9
bGH pA

FIG. 64



	Avg % Cutting		
	gRNA1	gRNA2	gRNA3
SCN2A	4.6	ND	2.2
KATNAL2	6.0	2.3	5.1
KCTD13	3.8	10.4	3.8
CHD8	ND	2.8*	8.1*
CACNA1C	9.4	3.9	6.3
CACNB2	5.1	1.2	3.0
GIT1	6.1	2.8	6.3
MECP2	2.2	2.7	6.3
SHANK3	20.2	9.8	7.8**

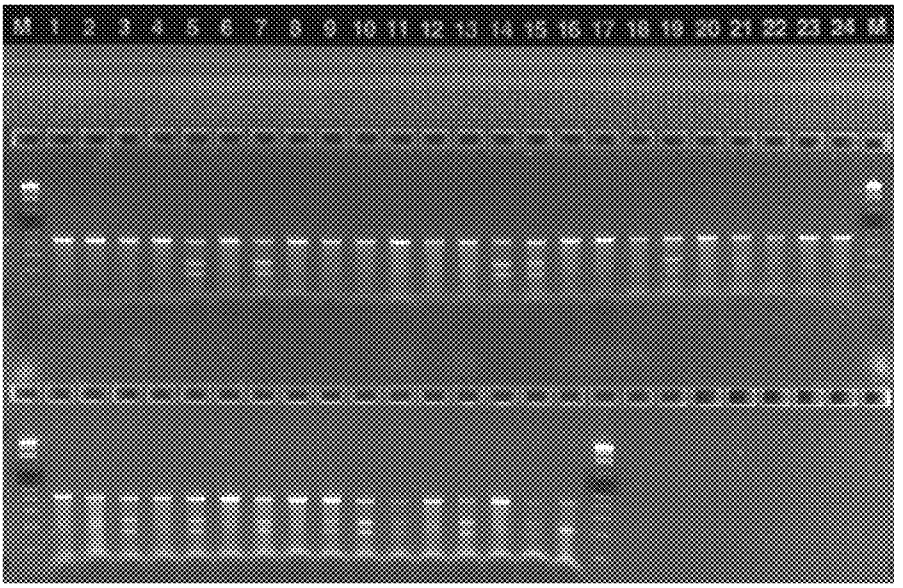
For injection, unique target

* Suboptimal gRNA, 1 off-target prediction

** Suboptimal gRNA, 7 off-target predictions

ND Not Determined

FIG. 65



gRNA sequences for Chd8 targeting:

Chd8.1 - agctgttttactggtcggct
Chd8.2 - aatggatacacctggtcgaa
Chd8.3 - caatggatacacctggtcga

FIG. 66

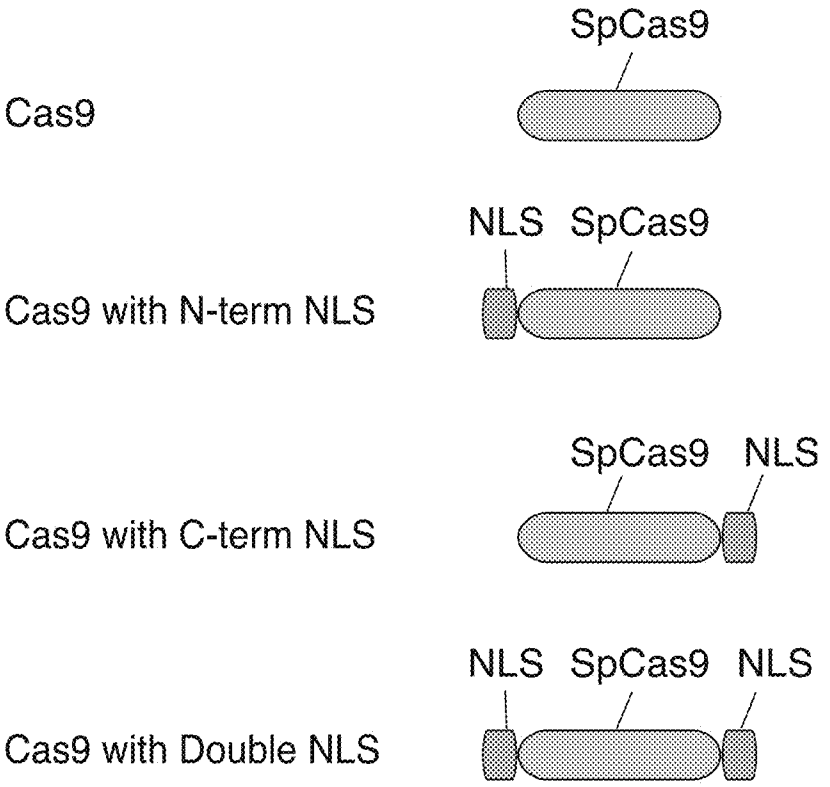


FIG. NLS1

FIG. 67

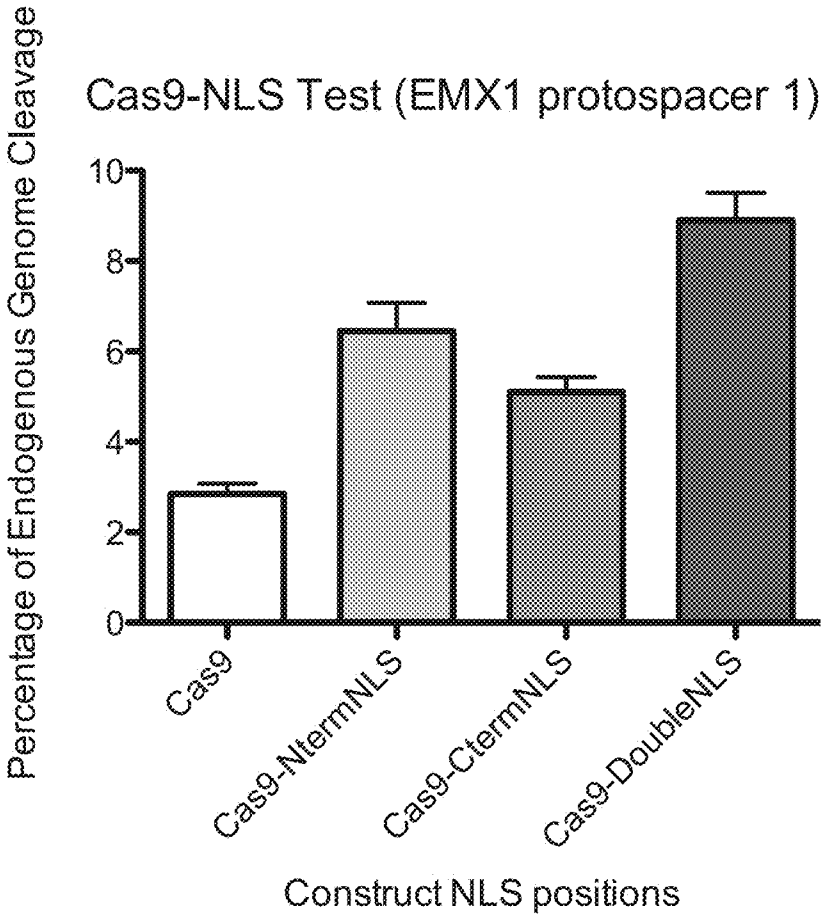
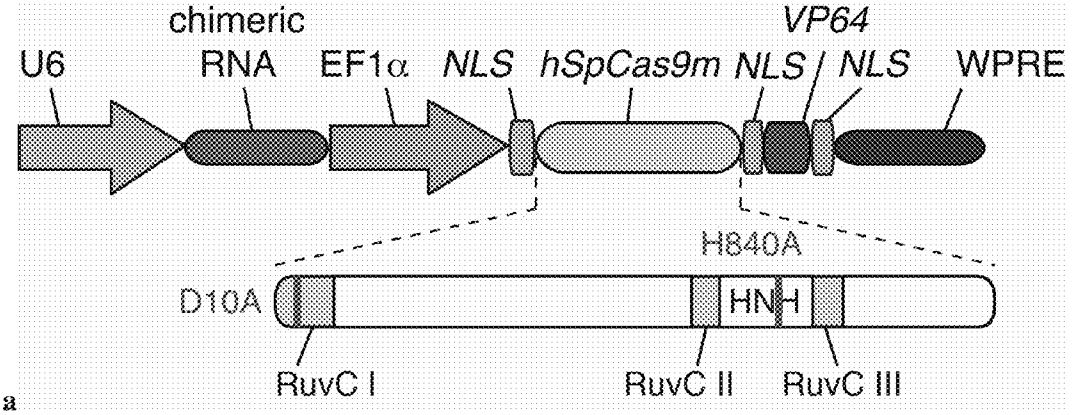
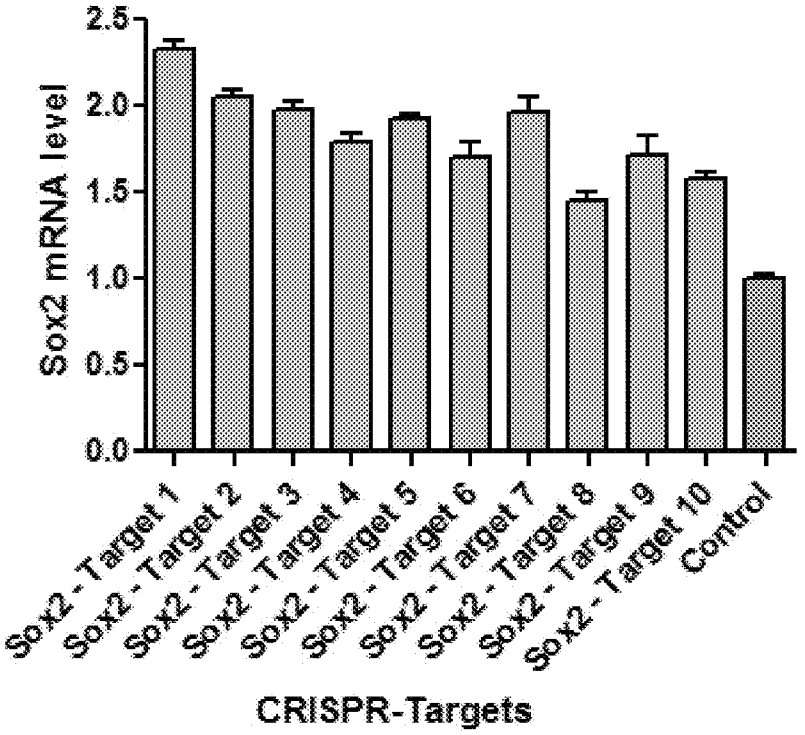

Cas9-NLS Test (EMX1 protospacer 1)
Percentage of Endogenous Genome Cleavage
10
8
6
4
2
0
Cas9
Cas9-NtermNLS
Cas9-CtermNLS
Cas9-DoubleNLS
Construct NLS positions

FIG. 68


chimeric
RNA
VP64
U6
EF1α
NLS
hSpCas9m
NLS
NLS
WPRE
H840A
D10A
HNH
RuvC I
RuvC II
RuvC III

a


Sox2 mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
Sox2 - Target 1
Sox2 - Target 2
Sox2 - Target 3
Sox2 - Target 4
Sox2 - Target 5
Sox2 - Target 6
Sox2 - Target 7
Sox2 - Target 8
Sox2 - Target 9
Sox2 - Target 10
Control
CRISPR-Targets

b

FIG. 69

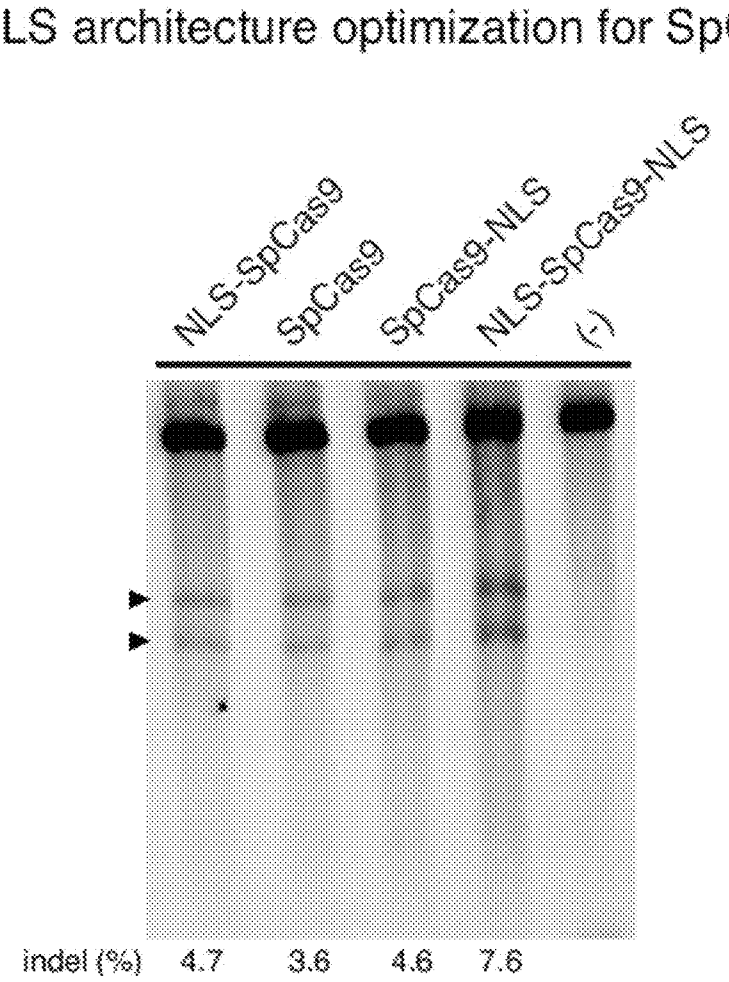
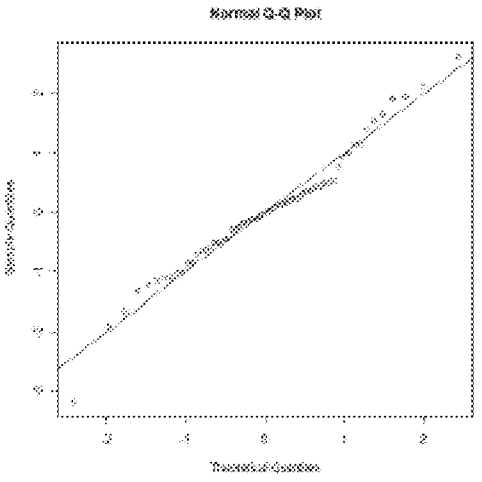

NLS architecture optimization for SpCas9
NLS-SpCas9
SpCas9
SpCas9-NLS
NLS-SpCas9-NLS
(-)
indel (%) 4.7 3.6 4.6 7.6

FIG. 70



QQ plot for the NGGNN sequences

FIG. 71

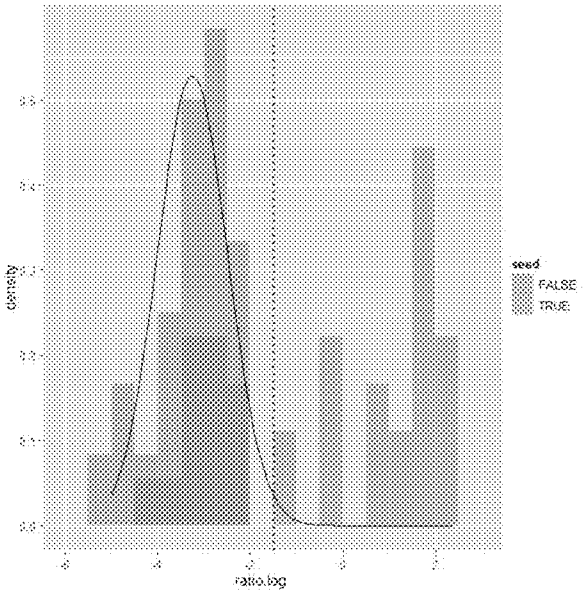


FIG. 72

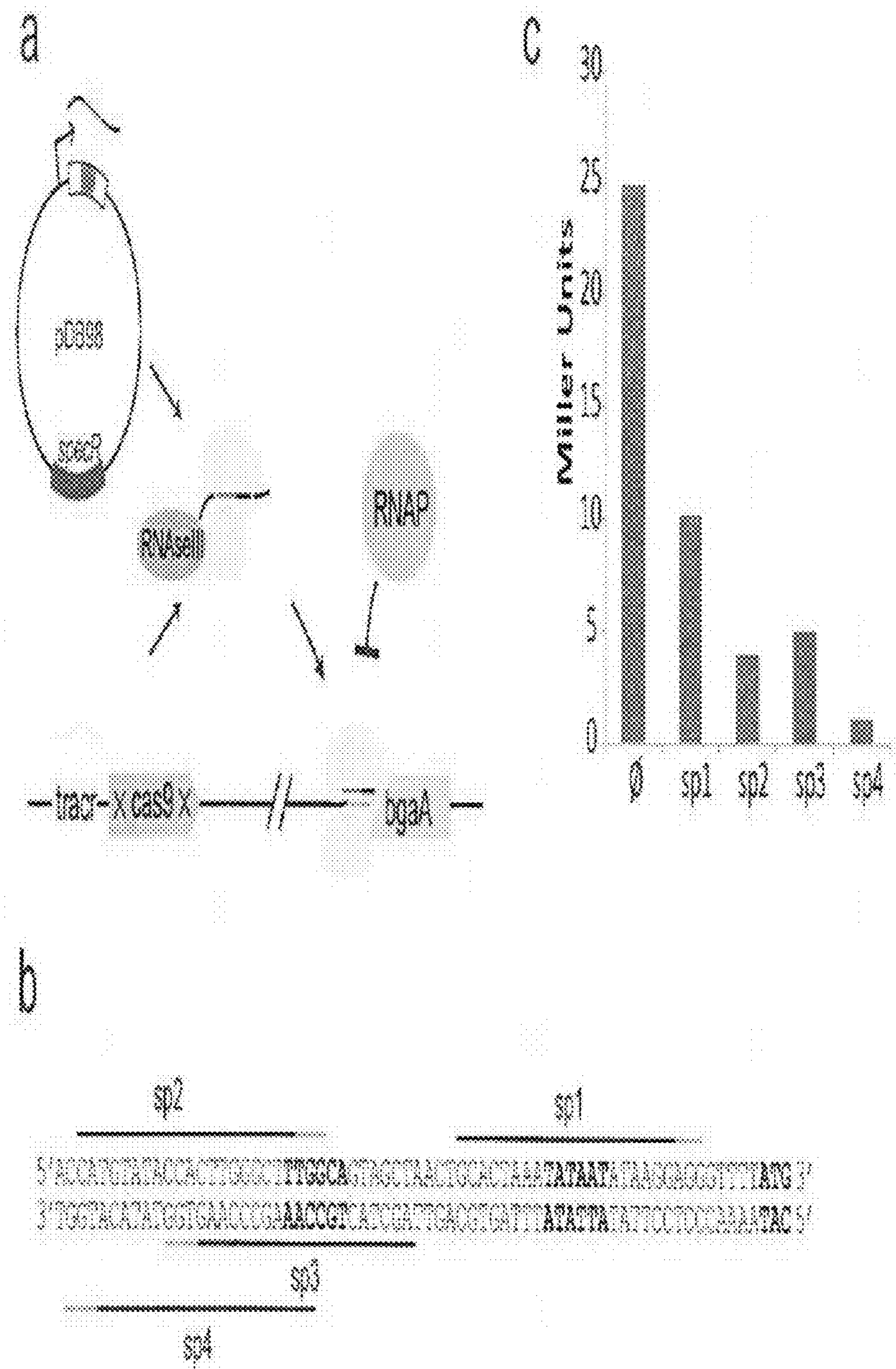

a
pDB98
specR
RNaseIII
RNAP
tracr
X cas9 X
bgaA
b
sp2
sp1
sp3
sp4
c
Miller Units
30
25
20
15
10
5
0
Ø
sp1
sp2
sp3
sp4

FIG. 73

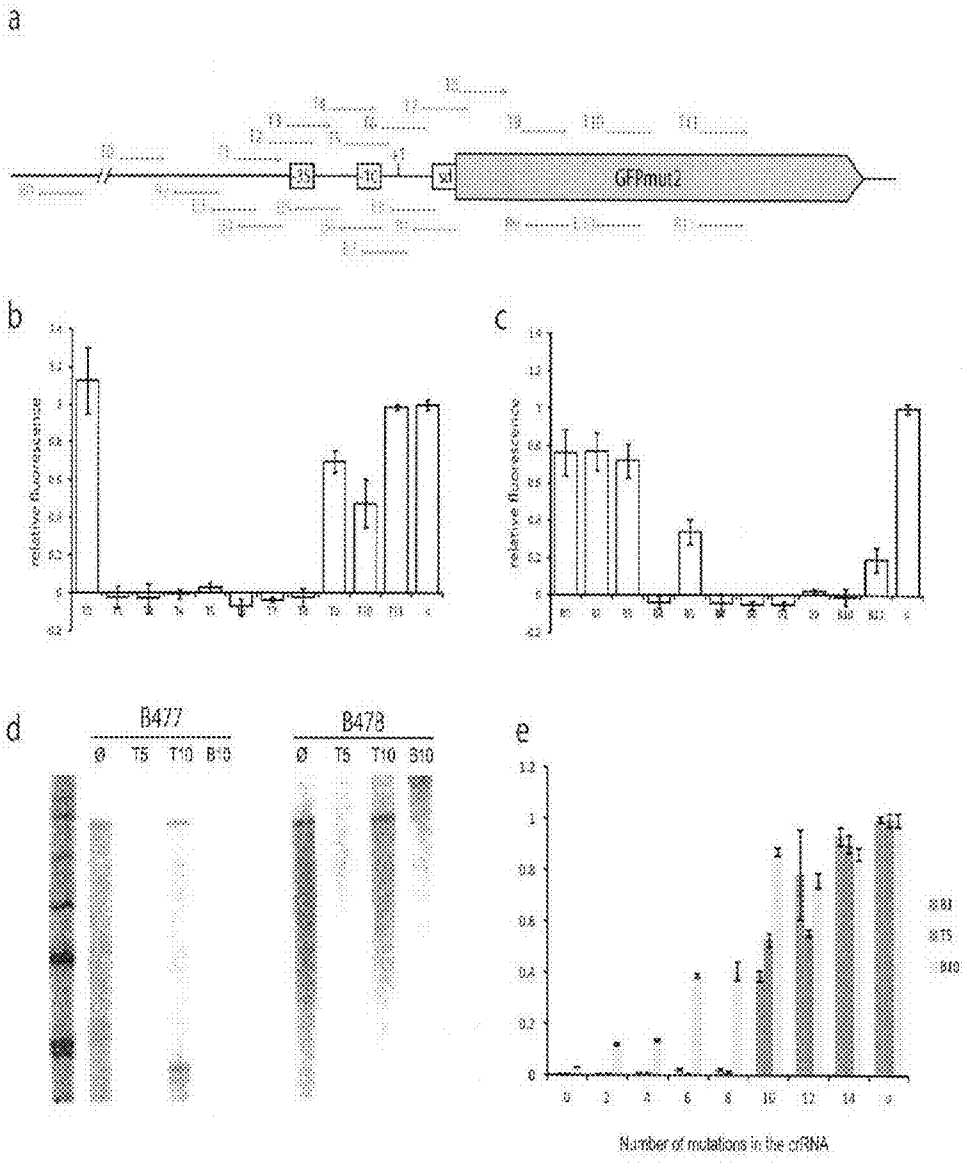

a
GFPmut2
b
relative fluorescence
c
relative fluorescence
d
B477
Ø T5 T10 B10
B478
Ø T5 T10 B10
e
Number of mutations in the crRNA

FIG. 74

CRISPR-CAS COMPONENT SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE

[0001] This application claims priority to U.S. provisional patent applications 61/736,527, 61/748,427, 61/768,959, 61/791,409 and 61/835,931 having Broad reference BI-2011/008/WSGR Docket No. 44063-701.101, BI-2011/008/WSGR Docket No. 44063-701.102, Broad reference BI-2011/008/VP Docket No. 44790.01.2003, BI-2011/008/VP Docket No. 44790.02.2003 and BI-2011/008/VP Docket No. 44790.03.2003 respectively, all entitled SYSTEMS METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION filed on Dec. 12, 2012, Jan. 2, 2013, Feb. 25, 2013, Mar. 15, 2013 and Jun. 17, 2013, respectively.

[0002] Reference is made to U.S. provisional patent application 61/758,468; 61/769,046; 61/802,174; 61/806,375; 61/814,263; 61/819,803 and 61/828,130, each entitled ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION, filed on Jan. 30, 2013; Feb. 25, 2013; Mar. 15, 2013; Mar. 28, 2013; Apr. 20, 2013; May 6, 2013 and May 28, 2013 respectively. Reference is also made to U.S. provisional patent application 61/835,936, 61/836,127, 61/836,101, 61/836,080, 61/836,123 and 61/835,973 each filed Jun. 17, 2013. Reference is also made to U.S. provisional patent application 61/842,322 and U.S. patent application Ser. No. 14/054,414, each having Broad reference BI-2011/008A, entitled CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS filed on Jul. 2, 2013 and Oct. 15, 2013 respectively.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0003] This invention was made with government support under the NIH Pioneer Award DP1 MH100706, awarded by the National Institutes of Health. The government has certain rights in the invention.

[0004] The foregoing applications, and all documents cited therein or during their prosecution ("appin cited documents") and all documents cited or referenced in the appin cited documents, and all documents cited or referenced herein ("herein cited documents"), and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

FIELD OF THE INVENTION

[0005] The present invention generally relates to systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that may use vector systems related to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and components thereof.

SEQUENCE LISTING

[0006] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Feb. 14, 2014, is named 44790.07.2003_SL.txt and is 305,328 bytes in size.

BACKGROUND OF THE INVENTION

[0007] Recent advances in genome sequencing techniques and analysis methods have significantly accelerated the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. Precise genome targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements, as well as to advance synthetic biology, biotechnological, and medical applications. Although genome-editing techniques such as designer zinc fingers, transcription activator-like effectors (TALEs), or homing meganucleases are available for producing targeted genome perturbations, there remains a need for new genome engineering technologies that are affordable, easy to set up, scalable, and amenable to targeting multiple positions within the eukaryotic genome.

SUMMARY OF THE INVENTION

[0008] There exists a pressing need for alternative and robust systems and techniques for sequence targeting with a wide array of applications. This invention addresses this need and provides related advantages. The CRISPR/Cas or the CRISPR-Cas system (both terms are used interchangeably throughout this application) does not require the generation of customized proteins to target specific sequences but rather a single Cas enzyme can be programmed by a short RNA molecule to recognize a specific DNA target, in other words the Cas enzyme can be recruited to a specific DNA target using said short RNA molecule. Adding the CRISPR-Cas system to the repertoire of genome sequencing techniques and analysis methods may significantly simplify the methodology and accelerate the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. To utilize the CRISPR-Cas system effectively for genome editing without deleterious effects, it is critical to understand aspects of engineering and optimization of these genome engineering tools, which are aspects of the claimed invention.

[0009] In one aspect, the invention provides a vector system comprising one or more vectors. In some embodiments, the system comprises: (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence; wherein components (a) and (b) are located on the same or different vectors of the system. In some embodiments, component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regu-

latory element. In some embodiments, component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In some embodiments, the system comprises the tracr sequence under the control of a third regulatory element, such as a polymerase III promoter. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. Determining optimal alignment is within the purview of one of skill in the art. For example, there are publically and commercially available alignment algorithms and programs such as, but not limited to, ClustalW, Smith-Waterman in matlab, Bowtie, Geneious, Biopython and SeqMan. In some embodiments, the CRISPR complex comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR complex in a detectable amount in the nucleus of a eukaryotic cell. Without wishing to be bound by theory, it is believed that a nuclear localization sequence is not necessary for CRISPR complex activity in eukaryotes, but that including such sequences enhances activity of the system, especially as to targeting nucleic acid molecules in the nucleus. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae, S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the CRISPR enzyme lacks DNA strand cleavage activity. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length. In general, and throughout this specification, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as "expression vectors." Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

[0010] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (e.g. in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell).

[0011] The term "regulatory element" is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g. transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (e.g. liver, pancreas), or particular cell types (e.g. lymphocytes). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In some embodiments, a vector comprises one or more pol III promoter (e.g. 1, 2, 3, 4, 5, or more pol III promoters), one or more pol II promoters (e.g. 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g. 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and H1 promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter. Also encompassed by the term "regulatory element" are enhancer elements, such as WPRE; CMV enhancers; the R-U5' segment in LTR of HTLV-I (Mol. Cell. Biol., Vol. 8(1), p. 466-472, 1988); SV40 enhancer; and the intron sequence between exons 2 and 3 of rabbit β-globin (Proc. Natl. Acad. Sci. USA., Vol. 78(3), p. 1527-31, 1981). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, etc. A vector can be introduced into host cells to thereby produce transcripts, proteins, or peptides, including fusion proteins or peptides, encoded by

nucleic acids as described herein (e.g., clustered regularly interspersed short palindromic repeats (CRISPR) transcripts, proteins, enzymes, mutant forms thereof, fusion proteins thereof, etc.).

[0012] Advantageous vectors include lentiviruses and adeno-associated viruses, and types of such vectors can also be selected for targeting particular types of cells.

[0013] In one aspect, the invention provides a vector comprising a regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising one or more nuclear localization sequences. In some embodiments, said regulatory element drives transcription of the CRISPR enzyme in a eukaryotic cell such that said CRISPR enzyme accumulates in a detectable amount in the nucleus of the eukaryotic cell. In some embodiments, the regulatory element is a polymerase II promoter. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae, S. pyogenes* or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the CRISPR enzyme lacks DNA strand cleavage activity.

[0014] In one aspect, the invention provides a CRISPR enzyme comprising one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae, S. pyogenes* or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme lacks the ability to cleave one or more strands of a target sequence to which it binds.

[0015] In one aspect, the invention provides a eukaryotic host cell comprising (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or, more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence. In some embodiments, the host cell comprises components (a) and (b). In some embodiments, component (a), component (b), or components (a) and (b) are stably integrated into a genome of the host eukaryotic cell. In some embodiments, component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element. In some embodiments, component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In some embodiments, the eukaryotic host cell further comprises a third regulatory element, such as a polymerase III promoter, operably linked to said tracr sequence. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. In some embodiments, the CRISPR enzyme comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae, S. pyogenes* or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the CRISPR enzyme lacks DNA strand cleavage activity. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length. In an aspect, the invention provides a non-human eukaryotic organism; preferably a multicellular eukaryotic organism, comprising a eukaryotic host cell according to any of the described embodiments. In other aspects, the invention provides a eukaryotic organism; preferably a multicellular eukaryotic organism, comprising a eukaryotic host cell according to any of the described embodiments. The organism in some embodiments of these aspects may be an animal; for example a mammal. Also, the organism may be an arthropod such as an insect. The organism also may be a plant. Further, the organism may be a fungus.

[0016] In one aspect, the invention provides a kit comprising one or more of the components described herein. In some embodiments, the kit comprises a vector system and instructions for using the kit. In some embodiments, the vector system comprises (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence. In some embodiments, the kit comprises components (a) and (b) located on the same or different vectors of the system. In some embodiments, component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element. In some embodiments, component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In

some embodiments, the system further comprises a third regulatory element, such as a polymerase III promoter, operably linked to said tracr sequence. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. In some embodiments, the CRISPR enzyme comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae, S. pyogenes* or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the CRISPR enzyme lacks DNA strand cleavage activity. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length.

[0017] In one aspect, the invention provides a method of modifying a target polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expressed from a gene comprising the target sequence. In some embodiments, the method further comprises delivering one or more vectors to said eukaryotic cell, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence. In some embodiments, said vectors are delivered to the eukaryotic cell in a subject. In some embodiments, said modifying takes place in said eukaryotic cell in a cell culture. In some embodiments, the method further comprises isolating said eukaryotic cell from a subject prior to said modifying. In some embodiments, the method further comprises returning said eukaryotic cell and/or cells derived therefrom to said subject.

[0018] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, the method further comprises delivering one or more vectors to said eukaryotic cells, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence.

[0019] In one aspect, the invention provides a method of generating a model eukaryotic cell comprising a mutated disease gene. In some embodiments, a disease gene is any gene associated an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) introducing one or more vectors into a eukaryotic cell, wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, and a tracr sequence; and (b) allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said disease gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, thereby generating a model eukaryotic cell comprising a mutated disease gene. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expression from a gene comprising the target sequence.

[0020] In one aspect, the invention provides a method for developing a biologically active agent that modulates a cell signaling event associated with a disease gene. In some embodiments, a disease gene is any gene associated an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) contacting a test compound with a model cell of any one of the described embodiments; and (b) detecting a change in a readout that is indicative of a reduction or an augmentation of a cell signaling event associated with said mutation in said disease gene, thereby developing said biologically active agent that modulates said cell signaling event associated with said disease gene.

[0021] In one aspect, the invention provides a recombinant polynucleotide comprising a guide sequence upstream of a tracr mate sequence, wherein the guide sequence when expressed directs sequence-specific binding of a CRISPR complex to a corresponding target sequence present in a eukaryotic cell. In some embodiments, the target sequence is a viral sequence present in a eukaryotic cell. In some embodiments, the target sequence is a proto-oncogene or an oncogene.

[0022] In one aspect the invention provides for a method of selecting one or more prokaryotic cell(s) by introducing one or more mutations in a gene in the one or more prokaryotic cell (s), the method comprising: introducing one or more vectors into the prokaryotic cell (s), wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, a tracr sequence, and a editing template; wherein the editing template comprises the one or more mutations that abolish CRISPR enzyme cleavage; allowing homologous recombination of the editing template with the target polynucleotide in the cell(s) to be selected; allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein binding of the CRISPR complex to the target polynucleotide induces cell death, thereby allowing one or more prokaryotic cell(s) in which one or more mutations have been introduced to be selected. In a preferred embodiment, the CRISPR enzyme is Cas9. In another aspect of the invention the cell to be selected may be a eukaryotic cell. Aspects of the invention allow for selection of specific cells without requiring a selection marker or a two-step process that may include a counter-selection system.

[0023] Accordingly, it is an object of the invention not to encompass within the invention any previously known product, process of making the product, or method of using the product such that Applicants reserve the right and hereby disclose a disclaimer of any previously known product, process, or method. It is further noted that the invention does not intend to encompass within the scope of the invention any product, process, or making of the product or method of using the product, which does not meet the written description and enablement requirements of the USPTO (35 U.S.C. §112, first paragraph) or the EPO (Article 83 of the EPC), such that Applicants reserve the right and hereby disclose a disclaimer of any previously described product, process of making the product, or method of using the product.

[0024] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention. These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0026] FIG. **1** shows a schematic model of the CRISPR system. The Cas9 nuclease from *Streptococcus pyogenes* (yellow) is targeted to genomic DNA by a synthetic guide RNA (sgRNA) consisting of a 20-nt guide sequence (blue) and a scaffold (red). The guide sequence base-pairs with the DNA target (blue), directly upstream of a requisite 5'-NGG protospacer adjacent motif (PAM; magenta), and Cas9 mediates a double-stranded break (DSB) ~3 bp upstream of the PAM (red triangle).

[0027] FIGS. **2**A-F show an exemplary CRISPR system, a possible mechanism of action, an example adaptation for expression in eukaryotic cells, and results of tests assessing nuclear localization and CRISPR activity. FIG. **2**C discloses SEQ ID NOS 279-280, respectively, in order of appearance. FIG. **2**E discloses SEQ ID NOS 281-283, respectively, in order of appearance. FIG. **2**F discloses SEQ ID NOS 284-288, respectively, in order of appearance.

[0028] FIG. **3** shows an exemplary expression cassette for expression of CRISPR system elements in eukaryotic cells, predicted structures of example guide sequences, and CRISPR system activity as measured in eukaryotic and prokaryotic cells (SEQ ID NOS 289-298, respectively, in order of appearance).

[0029] FIGS. **4**A-D show results of an evaluation of SpCas9 specificity for an example target. FIG. **4**A discloses SEQ ID NOS 299, 282 and 300-310, respectively, in order of appearance. FIG. **4**C discloses SEQ ID NO: 299.

[0030] FIGS. **5**A-G show an exemplary vector system and results for its use in directing homologous recombination in eukaryotic cells. FIG. **5**E discloses SEQ ID NO: 311. FIG. **5**F discloses SEQ ID NOS 312-313, respectively, in order of appearance. FIG. **5**G discloses SEQ ID NOS 314-318, respectively, in order of appearance.

[0031] FIG. **6** provides a table of protospacer sequences (SEQ ID NOS 33, 32, 31, 322-327, 35, 34 and 330-334, respectively, in order of appearance) and summarizes modification efficiency results for protospacer targets designed based on exemplary *S. pyogenes* and *S. thermophilus* CRISPR systems with corresponding PAMs against loci in human and mouse genomes. Cells were transfected with Cas9 and either pre-crRNA/tracrRNA or chimeric RNA, and analyzed 72 hours after transfection. Percent indels are calculated based on Surveyor assay results from indicated cell lines (N=3 for all protospacer targets, errors are S.E.M., N.D. indicates not detectable using the Surveyor assay, and N.T. indicates not tested in this study).

[0032] FIGS. **7**A-C show a comparison of different tracrRNA transcripts for Cas9-mediated gene targeting. FIG. **7**A discloses SEQ ID NOS 335-336, respectively, in order of appearance.

[0033] FIG. **8** shows a schematic of a surveyor nuclease assay for detection of double strand break-induced micro-insertions and -deletions.

[0034] FIGS. **9**A-B show exemplary bicistronic expression vectors for expression of CRISPR system elements in eukaryotic cells. FIG. **9**A discloses SEQ ID NOS 337-339, respectively, in order of appearance. FIG. **9**B discloses SEQ ID NOS 340-342, respectively, in order of appearance.

[0035] FIG. **10** shows a bacterial plasmid transformation interference assay, expression cassettes and plasmids used therein, and transformation efficiencies of cells used therein. FIG. **10**A discloses SEQ ID NOS 343-345, respectively, in order of appearance.

[0036] FIGS. **11**A-C show histograms of distances between adjacent *S. pyogenes* SF370 locus 1 PAM (NGG) (FIG. **10**A) and *S. thermophilus* LMD9 locus 2 PAM (NNA-

GAAW) (FIG. **10**B) in the human genome; and distances for each PAM by chromosome (Chi) (FIG. **10**C).

[0037] FIGS. **12**A-C show an exemplary CRISPR system, an example adaptation for expression in eukaryotic cells, and results of tests assessing CRISPR activity. FIG. **12**B discloses SEQ ID NOS 346-347, respectively, in order of appearance. FIG. **12**C discloses SEQ ID NO: 348.

[0038] FIGS. **13**A-C show exemplary manipulations of a CRISPR system for targeting of genomic loci in mammalian cells. FIG. **13**A discloses SEQ ID NO: 349. FIG. **13**B discloses SEQ ID NOS 350-352, respectively, in order of appearance.

[0039] FIGS. **14**A-B show the results of a Northern blot analysis of crRNA processing in mammalian cells. FIG. **14**A discloses SEQ ID NO: 353.

[0040] FIG. **15** shows an exemplary selection of protospacers in the human PVALB and mouse Th loci. FIG. **15**A discloses SEQ ID NO: 354. FIG. **15**B discloses SEQ ID NO: 355.

[0041] FIG. **16** shows example protospacer and corresponding PAM sequence targets of the *S. thermophilus* CRISPR system in the human EMX1 locus (SEQ ID NO: 348).

[0042] FIG. **17** provides a table of sequences for primers and probes (SEQ ID NOS 36-39 and 356-363, respectively, in order of appearance) used for Surveyor, RFLP, genomic sequencing, and Northern blot assays.

[0043] FIGS. **18**A-C show exemplary manipulation of a CRISPR system with chimeric RNAs and results of SURVEYOR assays for system activity in eukaryotic cells. FIG. **18**A discloses SEQ ID NO: 364, respectively, in order of appearance.

[0044] FIGS. **19**A-B show a graphical representation of the results of SURVEYOR assays for CRISPR system activity in eukaryotic cells (SEQ ID NOS 365-443, respectively, in order of appearance).

[0045] FIG. **20** shows an exemplary visualization of some *S. pyogenes* Cas9 target sites in the human genome using the UCSC genome browser.

[0046] FIG. **21** shows predicted secondary structures for exemplary chimeric RNAs comprising a guide sequence, tracr mate sequence, and tracr sequence (SEQ ID NOS 444-463, respectively, in order of appearance).

[0047] FIG. **22** shows exemplary bicistronic expression vectors for expression of CRISPR system elements in eukaryotic cells (SEQ ID NOS 464 and 341-342, respectively, in order of appearance).

[0048] FIG. **23** shows that Cas9 nuclease activity against endogenous targets may be exploited for genome editing. (a) Concept of genome editing using the CRISPR system. The CRISPR targeting construct directed cleavage of a chromosomal locus and was co-transformed with an editing template that recombined with the target to prevent cleavage. Kanamycin-resistant transformants that survived CRISPR attack contained modifications introduced by the editing template. tracr, trans-activating CRISPR RNA; aphA-3, kanamycin resistance gene. (b) Transformation of crR6M DNA in $R6^{8232.5}$ cells with no editing template, the R6 wild-type srtA or the R6370.1 editing templates. Recombination of either R6 srtA or $R6^{370.1}$ prevented cleavage by Cas9. Transformation efficiency was calculated as colony forming units (cfu) per μg of crR6M DNA; the mean values with standard deviations from at least three independent experiments are shown. PCR analysis was performed on 8 clones in each transformation. "Un." indicates the unedited srtA locus of strain $R6^{8232.5}$; "Ed." shows the editing template. $R6^{8232.5}$ and $R6^{370.1}$ targets are distinguished by restriction with EaeI.

[0049] FIG. **24** shows analysis of PAM and seed sequences that eliminate Cas9 cleavage. (a) PCR products with randomized PAM sequences or randomized seed sequences were transformed in crR6 cells (SEQ ID NOS 465-469, respectively, in order of appearance). These cells expressed Cas9 loaded with a crRNA that targeted a chromosomal region of $R6^{8232.5}$ cells (highlighted in pink) that is absent from the R6 genome. More than 2×105 chloramphenicol-resistant transformants, carrying inactive PAM or seed sequences, were combined for amplification and deep sequencing of the target region. (b) Relative proportion of number of reads after transformation of the random PAM constructs in crR6 cells (compared to number of reads in R6 transformants). The relative abundance for each 3-nucleotide PAM sequence is shown. Severely underrepresented sequences (NGG) are shown in red; partially underrepresented one in orange (NAG) (c) Relative proportion of number of reads after transformation of the random seed sequence constructs in crR6 cells (compared to number of reads in R6 transformants). The relative abundance of each nucleotide for each position of the first 20 nucleotides of the protospacer sequence is shown (SEQ ID NO: 470). High abundance indicates lack of cleavage by Cas9, i.e. a CRISPR inactivating mutation. The grey line shows the level of the WT sequence. The dotted line represents the level above which a mutation significantly disrupts cleavage (See section "Analysis of deep sequencing data" in Example 5)

[0050] FIG. **25** shows introduction of single and multiple mutations using the CRISPR system in *S. pneumoniae*. (a) Nucleotide and amino acid sequences of the wild-type and edited (green nucleotides; underlined amino acid residues) bgaA. The protospacer, PAM and restriction sites are shown (SEQ ID NOS 471-475 and 472, respectively, in order of appearance). (b) Transformation efficiency of cells transformed with targeting constructs in the presence of an editing template or control. (c) PCR analysis for 8 transformants of each editing experiment followed by digestion with BtgZI (R→*A) and TseI (NE→AA). Deletion of bgaA was revealed as a smaller PCR product. (d) Miller assay to measure the β-galactosidase activity of WT and edited strains. (e) For a single-step, double deletion the targeting construct contained two spacers (in this case matching srtA and bgaA) and was co-transformed with two different editing templates (f) PCR analysis for 8 transformants to detect deletions in srtA and bgaA loci. 6/8 transformants contained deletions of both genes.

[0051] FIG. **26** provides mechanisms underlying editing using the CRISPR system. (a) A stop codon was introduced in the erythromycin resistance gene ermAM to generate strain JEN53. The wild-type sequence can be restored by targeting the stop codon with the CRISPR::ermAM(stop) construct, and using the ermAM wild-type sequence as an editing template. (b) Mutant and wild-type ermAM sequences (SEQ ID NOS 476-479, respectively, in order of appearance). (c) Fraction of erythromicyn-resistant (erm^R) cfu calculated from total or kanamycin-resistant (kan^R) cfu. (d) Fraction of total cells that acquire both the CRISPR construct and the editing template. Co-transformation of the CRISPR targeting construct produced more transformants (t-test, p=0.011). In all cases the values show the mean±s.d. for three independent experiments.

[0052] FIG. **27** illustrates genome editing with the CRISPR system in *E. coli*. (a) A kanamycin-resistant plasmid carrying the CRISPR array (pCRISPR) targeting the gene to edit may be transformed in the HME63 recombineering strain containing a chloramphenicol-resistant plasmid harboring cas9 and tracr (pCas9), together with an oligonucleotide specifying the mutation. (b) A K42T mutation conferring streptomycin resistance was introduced in the rpsL gene (SEQ ID NOS 480-483, respectively, in order of appearance) (c) Fraction of streptomicyn-resistant ($strep^R$) cfu calculated from total or kanamycin-resistant (kan^R) cfu. (d) Fraction of total cells that acquire both the pCRISPR plasmid and the editing oligonucleotide. Co-transformation of the pCRISPR targeting plasmid produced more transformants (t-test, p=0.004). In all cases the values showed the mean±s.d. for three independent experiments.

[0053] FIG. **28** illustrates the transformation of crR6 genomic DNA leads to editing of the targeted locus (a) The IS1167 element of *S. pneumoniae* R6 was replaced by the CRISPR01 locus of *S. pyogenes* SF370 to generate crR6 strain. This locus encodes for the Cas9 nuclease, a CRISPR array with six spacers, the tracrRNA that is required for crRNA biogenesis and Cast, Cas2 and Csn2, proteins not necessary for targeting. Strain crR6M contains a minimal functional CRISPR system without cas1, cast and csn2. The aphA-3 gene encodes kanamycin resistance. Protospacers from the streptococcal bacteriophages ϕ8232.5 and ϕ370.1 were fused to a chloramphenicol resistance gene (cat) and integrated in the srtA gene of strain R6 to generate strains R68232.5 and R6370.1. (b) Left panel: Transformation of crR6 and crR6M genomic DNA in R68232.5 and R6370.1. As a control of cell competence a streptomycin resistant gene was also transformed. Right panel: PCR analysis of 8 $R6^{8232.5}$ transformants with crR6 genomic DNA. Primers that amplify the srtA locus were used for PCR. 7/8 genotyped colonies replaced the R68232.5 srtA locus by the WT locus from the crR6 genomic DNA.

[0054] FIG. **29** provides chromatograms of DNA sequences of edited cells obtained in this study. In all cases the wild-type and mutant protospacer and PAM sequences (or their reverse complement) are indicated. When relevant, the amino acid sequence encoded by the protospacer is provided. For each editing experiment, all strains for which PCR and restriction analysis corroborated the introduction of the desired modification were sequenced. A representative chromatogram is shown. (a) Chromatogram for the introduction of a PAM mutation into the $R6^{8232.5}$ target (FIG. **23***d*) (SEQ ID NOS 484-485, respectively, in order of appearance). (b) Chromatograms for the introduction of the R>A and NE>AA mutations into β-galactosidase (bgaA) (FIG. **25***c*) (SEQ ID NOS 471-475 and 472, respectively, in order of appearance). (c) Chromatogram for the introduction of a 6664 bp deletion within bgaA ORF (FIGS. **25***c* and **25***f*). The dotted line indicates the limits of the deletion (SEQ ID NOS 486-488, respectively, in order of appearance). (d) Chromatogram for the introduction of a 729 bp deletion within srtA ORF (FIG. **25***f*). The dotted line indicates the limits of the deletion (SEQ ID NOS 489-491, respectively, in order of appearance). (e) Chromatograms for the generation of a premature stop codon within ermAM (FIG. **33**) (SEQ ID NOS 492-495, respectively, in order of appearance). (f) rpsL editing in *E. coli* (FIG. **27**) (SEQ ID NOS 480-483, respectively, in order of appearance).

[0055] FIG. **30** illustrates CRISPR immunity against random *S. pneumoniae* targets containing different PAMs. (a) Position of the 10 random targets on the *S. pneumoniae* R6 genome. The chosen targets have different PAMs and are on both strands. (b) Spacers corresponding to the targets were cloned in a minimal CRISPR array on plasmid pLZ12 and transformed into strain crR6Rc, which supplies the processing and targeting machinery in trans. (c) Transformation efficiency of the different plasmids in strain R6 and crR6Rc. No colonies were recovered for the transformation of pDB99-108 (T1-T10) in crR6Rc. The dashed line represents limit of detection of the assay.

[0056] FIG. **31** provides a general scheme for targeted genome editing. To facilitate targeted genome editing, crR6M was further engineered to contain tracrRNA, Cas9 and only one repeat of the CRISPR array followed by kanamycin resistance marker (aphA-3), generating strain crR6Rk. DNA from this strain is used as a template for PCR with primers designed to introduce a new spacer (green box designated with N). The left and right PCRs are assembled using the Gibson method to create the targeting construct. Both the targeting and editing constructs are then transformed into strain crR6Rc, which is a strain equivalent to crR6Rk but has the kanamycin resistance marker replaced by a chloramphenicol resistance marker (cat). About 90% of the kanamycin-resistant transformants contain the desired mutation.

[0057] FIG. **32** illustrates the distribution of distances between PAMs. NGG and CCN that are considered to be valid PAMs. Data is shown for the *S. pneumoniae* R6 genome as well as for a random sequence of the same length and with the same GC-content (39.7%). The dotted line represents the average distance (12) between PAMs in the R6 genome.

[0058] FIG. **33** illustrates CRISPR-mediated editing of the ermAM locus using genomic DNA as targeting construct. To use genomic DNA as targeting construct it is necessary to avoid CRISPR autoimmunity, and therefore a spacer against a sequence not present in the chromosome must be used (in this case the ermAM erythromycin resistance gene). (a) Nucleotide and amino acid sequences of the wild-type and mutated (red letters) ermAM gene. The protospacer and PAM sequences are shown (SEQ ID NOS 492-495, respectively, in order of appearance). (b) A schematic for CRISPR-mediated editing of the ermAM locus using genomic DNA. A construct carrying an ermAM-targeting spacer (blue box) is made by PCR and Gibson assembly, and transformed into strain crR6Rc, generating strain JEN37. The genomic DNA of JEN37 was then used as a targeting construct, and was co-transformed with the editing template into JEN38, a strain in which the srtA gene was replaced by a wild-type copy of ermAM. Kanamycin-resistant transformants contain the edited genotype (JEN43). (c) Number of kanamycin-resistant cells obtained after co-transformation of targeting and editing or control templates. In the presence of the control template 5.4×10^3 cfu/ml were obtained, and 4.3×10^5 cfu/ml when the editing template was used. This difference indicates an editing efficiency of about 99% [$(4.3\times10^5-5.4\times10^3)/4.3\times10^5$]. (d) To check for the presence of edited cells seven kanamycin-resistant clones and JEN38 were streaked on agar plates with (erm+) or without (erm−) erythromycin. Only the positive control displayed resistance to erythromycin. The ermAM mut genotype of one of these transformants was also verified by DNA sequencing (FIG. **29***e*).

[0059] FIG. **34** illustrates sequential introduction of mutations by CRISPR-mediated genome editing. (a) A schematic

for sequential introduction of mutations by CRISPR-mediated genome editing. First, R6 is engineered to generate crR6Rk. crR6Rk is co-transformed with a srtA-targeting construct fused to cat for chloramphenicol selection of edited cells, along with an editing construct for a ΔsrtA in-frame deletion. Strain crR6 ΔsrtA is generated by selection on chloramphenicol. Subsequently, the ΔsrtA strain is co-transformed with a bgaA-targeting construct fused to aphA-3 for kanamycin selection of edited cells, and an editing construct containing a ΔbgaA in-frame deletion. Finally, the engineered CRISPR locus can be erased from the chromosome by first co-transforming R6DNA containing the wild-type IS1167 locus and a plasmid carrying a bgaA protospacer (pDB97), and selection on spectinomycin. (b) PCR analysis for 8 chloramphenicol (Cam)-resistant transformants to detect the deletion in the srtA locus. (c) β-galactosidase activity as measured by Miller assay. In *S. pneumoniae*, this enzyme is anchored to the cell wall by sortase A. Deletion of the srtA gene results in the release of β-galactosidase into the supernatant. ΔbgaA mutants show no activity. (d) PCR analysis for 8 spectinomycin (Spec)-resistant transformants to detect the replacement of the CRISPR locus by wild-type IS1167.

[0060] FIG. **35** illustrates the background mutation frequency of CRISPR in *S. pneumoniae*. (a) Transformation of the CRISPR::Ø or CRISPR::erm(stop) targeting constructs in JEN53, with or without the ermAM editing template. The difference in kan^R CFU between CRISPR::Ø and CRISPR::erm(stop) indicates that Cas9 cleavage kills non-edited cells. Mutants that escape CRISPR interference in the absence of editing template are observed at a frequency of 3×10^{-3}. (b) PCR analysis of the CRISPR locus of escapers shows that 7/8 have a spacer deletion. (c) Escaper #2 carries a point mutation in cas9 (SEQ ID NOS 496-499, respectively, in order of appearance).

[0061] FIG. **36** illustrates that the essential elements of the *S. pyogenes* CRISPR locus 1 are reconstituted in *E. coli* using pCas9. The plasmid contained tracrRNA, Cas9, as well as a leader sequence driving the crRNA array. The pCRISPR plasmids contained the leader and the array only. Spacers may be inserted into the crRNA array between BsaI sites using annealed oligonucleotides (SEQ ID NOS 343, 500 and 127, respectively, in order of appearance). Oligonucleotide design is shown at bottom. pCas9 carried chloramphenicol resistance (CmR) and is based on the low-copy pACYC184 plasmid backbone. pCRISPR is based on the high-copy number pZE21 plasmid. Two plasmids were required because a pCRISPR plasmid containing a spacer targeting the *E. coli* chromosome may not be constructed using this organism as a cloning host if Cas9 is also present (it will kill the host).

[0062] FIG. **37** illustrates CRISPR-directed editing in *E. coli* MG1655. An oligonucleotide (W542) carrying a point mutation that both confers streptomycin resistance and abolishes CRISPR immunity, together with a plasmid targeting rpsL (pCRISPR::rpsL) or a control plasmid (pCRISPR::Ø) were co-transformed into wild-type *E. coli* strain MG1655 containing pCas9. Transformants were selected on media containing either streptomycin or kanamycin. Dashed line indicates limit of detection of the transformation assay.

[0063] FIG. **38** illustrates the background mutation frequency of CRISPR in *E. coli* HME63. (a) Transformation of the pCRISPR::Ø or pCRISPR::rpsL plasmids into HME63 competent cells. Mutants that escape CRISPR interference were observed at a frequency of 2.6×10^{-4}. (b) Amplification of the CRISPR array of escapers showed that 8/8 have deleted the spacer.

[0064] FIGS. **39**A-D show a circular depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[0065] FIGS. **40**A-F show the linear depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[0066] FIG. **41**A-M shows sequences where the mutation points are located within the SpCas9 gene (SEQ ID NOS 501-502, respectively, in order of appearance).

[0067] FIG. **42** shows a schematic construct in which the transcriptional activation domain (VP64) is fused to Cas9 with two mutations in the catalytic domains (D10 and H840).

[0068] FIG. **43**A-D shows genome editing via homologous recombination. (a) Schematic of SpCas9 nickase, with D10A mutation in the RuvC I catalytic domain. (b) Schematic representing homologous recombination (HR) at the human EMX1 locus using either sense or antisense single stranded oligonucleotides as repair templates. Red arrow above indicates sgRNA cleavage site; PCR primers for genotyping (Tables J and K) are indicated as arrows in right panel. (c) Sequence of region modified by HR. d, SURVEYOR assay for wildtype (wt) and nickase (D10A) SpCas9-mediated indels at the EMX1 target 1 locus (n=3) (SEQ ID NOS 503-505, 503, 506 and 505, respectively, in order of appearance). Arrows indicate positions of expected fragment sizes.

[0069] FIGS. **44**A-B show single vector designs for SpCas9. FIG. **44**A discloses SEQ ID NOS 320-321 and 328, respectively, in order of appearance. FIG. **44**B discloses SEQ ID NO: 329.

[0070] FIG. **45** shows quantification of cleavage of NLS-Csn1 constructs NLS-Csn1, Csn1, Csn1-NLS, NLS-Csn1-NLS, NLS-Csn1-GFP-NLS and UnTFN.

[0071] FIG. **46** shows index frequency of NLS-Cas9, Cas9, Cas9-NLS and NLS-Cas9-NLS.

[0072] FIG. **47** shows a gel demonstrating that SpCas9 with nickase mutations (individually) do not induce double strand breaks.

[0073] FIG. **48** shows a design of the oligo DNA used as Homologous Recombination (HR) template in this experiment and a comparison of HR efficiency induced by different combinations of Cas9 protein and HR template.

[0074] FIG. **49**A shows the Conditional Cas9, *Rosa*26 targeting vector map.

[0075] FIG. **49**B shows the Constitutive Cas9, *Rosa*26 targeting vector map.

[0076] FIG. **50**A-H show the sequences of each element present in the vector maps of FIGS. **49**A-B (SEQ ID NOS 507-516, respectively, in order of appearance).

[0077] FIG. **51** shows a schematic of the important elements in the Constitutive and Conditional Cas9 constructs.

[0078] FIG. **52** shows the functional validation of the expression of Constitutive and Conditional Cas9 constructs.

[0079] FIG. **53** shows the validation of Cas9 nuclease activity by Surveyor.

[0080] FIG. **54** shows the quantification of Cas9 nuclease activity.

[0081] FIG. **55** shows construct design and homologous recombination (HR) strategy.

[0082] FIG. **56** shows the genomic PCR genotyping results for the constitutive (Right) and conditional (Left) constructs at two different gel exposure times (top row for 3 min and bottom row for 1 min).

[0083] FIG. **57** shows Cas9 activation in mESCs.

[0084] FIG. **58** shows a schematic of the strategy used to mediate gene knockout via NHEJ using a nickase version of Cas9 along with two guide RNAs.

[0085] FIG. **59** shows how DNA double-strand break (DSB) repair promotes gene editing. In the error-prone non-homologous end joining (NHEJ) pathway, the ends of a DSB are processed by endogenous DNA repair machineries and rejoined together, which can result in random insertion/deletion (indel) mutations at the site of junction. Indel mutations occurring within the coding region of a gene can result in frame-shift and a premature stop codon, leading to gene knockout. Alternatively, a repair template in the form of a plasmid or single-stranded oligodeoxynucleotides (ssODN) can be supplied to leverage the homology-directed repair (HDR) pathway, which allows high fidelity and precise editing.

[0086] FIG. **60** shows the timeline and overview of experiments. Steps for reagent design, construction, validation, and cell line expansion. Custom sgRNAs (light blue bars) for each target, as well as genotyping primers, are designed in silico via our online design tool (available at the website genome-engineering.org/tools). sgRNA expression vectors are then cloned into a plasmid containing Cas9 (PX330) and verified via DNA sequencing. Completed plasmids (pCRISPRs), and optional repair templates for facilitating homology directed repair, are then transfected into cells and assayed for ability to mediate targeted cleavage. Finally, transfected cells can be clonally expanded to derive isogenic cell lines with defined mutations.

[0087] FIG. **61**A-C shows Target selection and reagent preparation. (a) For *S. pyogenes* Cas9, 20-bp targets (highlighted in blue) must be followed by 5'-NGG, which can occur in either strand on genomic DNA. We recommend using the online tool described in this protocol in aiding target selection (www.genome-engineering.org/tools). (b) Schematic for co-transfection of Cas9 expression plasmid (PX165) and PCR-amplified U6-driven sgRNA expression cassette. Using a U6 promoter-containing PCR template and a fixed forward primer (U6 Fwd), sgRNA-encoding DNA can appended onto the U6 reverse primer (U6 Rev) and synthesized as an extended DNA oligo (Ultramer oligos from IDT). Note the guide sequence (blue N's) in U6 Rev is the reverse complement of the 5'-NGG flanking target sequence (SEQ ID NOS 517 and 517-519, respectively, in order of appearance). (c) Schematic for scarless cloning of the guide sequence oligos into a plasmid containing Cas9 and sgRNA scaffold (PX330). The guide oligos (blue N's) contain overhangs for ligation into the pair of BbsI sites on PS330, with the top and bottom strand orientations matching those of the genomic target (i.e. top oligo is the 20-bp sequence preceding 5'-NGG in genomic DNA). Digestion of PX330 with BbsI allows the replacement of the Type IIs restriction sites (blue outline) with direct insertion of annealed oligos. It is worth noting that an extra G was placed before the first base of the guide sequence. Applicants have found that an extra G in front of the guide sequence does not adversely affect targeting efficiency. In cases when the 20-nt guide sequence of choice does not begin with guanine, the extra guanine will ensure the sgRNA is efficiently transcribed by the U6 promoter, which prefers a guanine in the first base of the transcript (SEQ ID NOS 320-321 and 328, respectively, in order of appearance).

[0088] FIG. **62**A-D shows the anticipated results for multiplex NHEJ. (a) Schematic of the SURVEYOR assay used to determine indel percentage. First, genomic DNA from the heterogeneous population of Cas9-targeted cells is amplified by PCR. Amplicons are then reannealed slowly to generate heteroduplexes. The reannealed heteroduplexes are cleaved by SURVEYOR nuclease, whereas homoduplexes are left intact. Cas9-mediated cleavage efficiency (% indel) is calculated based on the fraction of cleaved DNA, as determined by integrated intensity of gel bands. (b) Two sgRNAs (orange and blue bars) are designed to target the human GRIN2B and DYRK1A loci. SURVEYOR gel shows modification at both loci in transfected cells. Colored arrows indicated expected fragment sizes for each locus. (c) A pair of sgRNAs (light blue and green bars) are designed to excise an exon (dark blue) in the human EMX1 locus. Target sequences and PAMs (red) are shown in respective colors, and sites of cleavage indicated by red triangle. Predicted junction is shown below. Individual clones isolated from cell populations transfected with sgRNA 3, 4, or both are assayed by PCR (OUT Fwd, OUT Rev), reflecting a deletion of ~270-bp. Representative clones with no modification (12/23), mono-allelic (10/23), and bi-allelic (1/23) modifications are shown. IN Fwd and IN Rev primers are used to screen for inversion events (FIG. **6***d*) (SEQ ID NOS 520-522, respectively, in order of appearance). (d) Quantification of clonal lines with EMX1 exon deletions. Two pairs of sgRNAs (3.1, 3.2 left-flanking sgRNAs; 4.1, 4.2, right flanking sgRNAs) are used to mediate deletions of variable sizes around one EMX1 exon. Transfected cells are clonally isolated and expanded for genotyping analysis for deletions and inversion events. Of the 105 clones are screened, 51 (49%) and 11 (10%) carrying heterozygous and homozygous deletions, respectively. Approximate deletion sizes are given since junctions may be variable.

[0089] FIG. **63**A-C shows the application of ssODNs and targeting vector to mediate HR with both wildtype and nickase mutant of Cas9 in HEK293FT and HUES9 cells with efficiencies ranging from 1.0-27%. FIG. **63**B discloses SEQ ID NOS 503-505, 503, 506 and 505, respectively, in order of appearance.

[0090] FIG. **64** shows a schematic of a PCR-based method for rapid and efficient CRISPR targeting in mammalian cells. A plasmid containing the human RNA polymerase III promoter U6 is PCR-amplified using a U6-specific forward primer and a reverse primer carrying the reverse complement of part of the U6 promoter, the sgRNA(+85) scaffold with guide sequence, and 7 T nucleotides for transcriptional termination. The resulting PCR product is purified and co-delivered with a plasmid carrying Cas9 driven by the CBh promoter (SEQ ID NOS 517, 523, 518 and 524-525, respectively, in order of appearance).

[0091] FIG. **65** shows SURVEYOR Mutation Detection Kit from Transgenomics results for each gRNA and respective controls. A positive SURVEYOR result is one large band corresponding to the genomic PCR and two smaller bands that are the product of the SURVEYOR nuclease making a double-strand break at the site of a mutation. Each gRNA was validated in the mouse cell line, Neuro-N2a, by liposomal transient co-transfection with hSpCas9. 72 hours post-transfection genomic DNA was purified using QuickExtract DNA from Epicentre. PCR was performed to amplify the locus of interest.

[0092] FIG. **66** shows Surveyor results for 38 live pups (lanes 1-38) 1 dead pup (lane 39) and 1 wild-type pup for comparison (lane 40). Pups 1-19 were injected with gRNA Chd8.2 and pups 20-38 were injected with gRNA Chd8.3. Of the 38 live pups, 13 were positive for a mutation. The one dead pup also had a mutation. There was no mutation detected in the wild-type sample. Genomic PCR sequencing was consistent with the SURVEYOR assay findings (SEQ ID NOS 526-528, respectively, in order of appearance).

[0093] FIG. **67** shows a design of different Cas9 NLS constructs. All Cas9 were the human-codon-optimized version of the Sp Cas9. NLS sequences are linked to the cas9 gene at either N-terminus or C-terminus. All Cas9 variants with different NLS designs were cloned into a backbone vector containing so it is driven by EF1a promoter. On the same vector there is a chimeric RNA targeting human EMX1 locus driven by U6 promoter, together forming a two-component system.

[0094] FIG. **68** shows the efficiency of genomic cleavage induced by Cas9 variants bearing different NLS designs. The percentage indicate the portion of human EMX1 genomic DNA that were cleaved by each construct. All experiments are from 3 biological replicates. n=3, error indicates S.E.M.

[0095] FIG. **69**A shows a design of the CRISPR-TF (Transcription Factor) with transcriptional activation activity. The chimeric RNA is expressed by U6 promoter, while a human-codon-optimized, double-mutant version of the Cas9 protein (hSpCas9m), operably linked to triple NLS and a VP64 functional domain is expressed by a EF1a promoter. The double mutations, D10A and H840A, renders the cas9 protein unable to introduce any cleavage but maintained its capacity to bind to target DNA when guided by the chimeric RNA.

[0096] FIG. **69**B shows transcriptional activation of the human SOX2 gene with CRISPR-TF system (Chimeric RNA and the Cas9-NLS-VP64 fusion protein). 293FT cells were transfected with plasmids bearing two components: (1) U6-driven different chimeric RNAs targeting 20-bp sequences within or around the human SOX2 genomic locus, and (2) EF1a-driven hSpCas9m (double mutant)-NLS-VP64 fusion protein. 96 hours post transfection, 293FT cells were harvested and the level of activation is measured by the induction of mRNA expression using a qRT-PCR assay. All expression levels are normalized against the control group (grey bar), which represents results from cells transfected with the CRISPR-TF backbone plasmid without chimeric RNA. The qRT-PCR probes used for detecting the SOX2 mRNA is Taqman Human Gene Expression Assay (Life Technologies). All experiments represents data from 3 biological replicates, n=3, error bars show s.e.m.

[0097] FIG. **70** depicts NLS architecture optimization for SpCas9.

[0098] FIG. **71** shows a QQ plot for NGGNN sequences.

[0099] FIG. **72** shows a histogram of the data density with fitted normal distribution (black line) and 0.99 quantile (dotted line).

[0100] FIG. **73**A-C shows RNA-guided repression of bgaA expression by dgRNA::cas9**. a. The Cas9 protein binds to the tracrRNA, and to the precursor CRISPR RNA which is processed by RNAseIII to form the crRNA. The crRNA directs binding of Cas9 to the bgaA promoter and represses transcription. b. The targets used to direct Cas9** to the bgaA promoter are represented (SEQ ID NO: 529). Putative −35, −10 as well as the bgaA start codon are in bold. c. Betagalactosidase activity as measure by Miller assay in the absence of targeting and for the four different targets.

[0101] FIG. **74**A-E shows characterization of Cas9** mediated repression. a. The gfpmut2 gene and its promoter, including the −35 and −10 signals are represented together with the position of the different target sites used the study. b. Relative fluorescence upon targeting of the coding strand. c. Relative fluorescence upon targeting of the non-coding strand. d. Northern blot with probes B477 and B478 on RNA extracted from T5, T10, B10 or a control strain without a target. e. Effect of an increased number of mutations in the 5' end of the crRNA of B1, T5 and B10.

[0102] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE INVENTION

[0103] The terms "polynucleotide", "nucleotide", "nucleotide sequence", "nucleic acid" and "oligonucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

[0104] In aspects of the invention the terms "chimeric RNA", "chimeric guide RNA", "guide RNA", "single guide RNA" and "synthetic guide RNA" are used interchangeably and refer to the polynucleotide sequence comprising the guide sequence, the tracr sequence and the tracr mate sequence. The term "guide sequence" refers to the about 20 bp sequence within the guide RNA that specifies the target site and may be used interchangeably with the terms "guide" or "spacer". The term "tracr mate sequence" may also be used interchangeably with the term "direct repeat(s)".

[0105] As used herein the term "wild type" is a term of the art understood by skilled persons and means the typical form of an organism, strain, gene or characteristic as it occurs in nature as distinguished from mutant or variant forms.

[0106] As used herein the term "variant" should be taken to mean the exhibition of qualities that have a pattern that deviates from what occurs in nature.

[0107] The terms "non-naturally occurring" or "engineered" are used interchangeably and indicate the involvement of the hand of man. The terms, when referring to nucleic acid molecules or polypeptides mean that the nucleic acid molecule or the polypeptide is at least substantially free from at least one other component with which they are naturally associated in nature and as found in nature.

[0108] "Complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick base pairing or

other non-traditional types. A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. "Substantially complementary" as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions.

[0109] As used herein, "stringent conditions" for hybridization refer to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent, and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions are described in detail in Tijssen (1993), Laboratory Techniques In Biochemistry And Molecular Biology-Hybridization With Nucleic Acid Probes Part I, Second Chapter "Overview of principles of hybridization and the strategy of nucleic acid probe assay", Elsevier, N.Y.

[0110] "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogstein binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PCR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the "complement" of the given sequence.

[0111] As used herein, "expression" refers to the process by which a polynucleotide is transcribed from a DNA template (such as into and mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as "gene product." If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[0112] The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term "amino acid" includes natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics.

[0113] The terms "subject," "individual," and "patient" are used interchangeably herein to refer to a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets. Tissues, cells and their progeny of a biological entity obtained in vivo or cultured in vitro are also encompassed.

[0114] The terms "therapeutic agent", "therapeutic capable agent" or "treatment agent" are used interchangeably and refer to a molecule or compound that confers some beneficial effect upon administration to a subject. The beneficial effect includes enablement of diagnostic determinations; amelioration of a disease, symptom, disorder, or pathological condition; reducing or preventing the onset of a disease, symptom, disorder or condition; and generally counteracting a disease, symptom, disorder or pathological condition.

[0115] As used herein, "treatment" or "treating," or "palliating" or "ameliorating" are used interchangeably. These terms refer to an approach for obtaining beneficial or desired results including but not limited to a therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant any therapeutically relevant improvement in or effect on one or more diseases, conditions, or symptoms under treatment. For prophylactic benefit, the compositions may be administered to a subject at risk of developing a particular disease, condition, or symptom, or to a subject reporting one or more of the physiological symptoms of a disease, even though the disease, condition, or symptom may not have yet been manifested.

[0116] The term "effective amount" or "therapeutically effective amount" refers to the amount of an agent that is sufficient to effect beneficial or desired results. The therapeutically effective amount may vary depending upon one or more of: the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. The term also applies to a dose that will provide an image for detection by any one of the imaging methods described herein. The specific dose may vary depending on one or more of: the particular agent chosen, the dosing regimen to be followed, whether it is administered in combination with other compounds, timing of administration, the tissue to be imaged, and the physical delivery system in which it is carried.

[0117] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R. I. Freshney, ed. (1987)).

[0118] Several aspects of the invention relate to vector systems comprising one or more vectors, or vectors as such.

Vectors can be designed for expression of CRISPR transcripts (e.g. nucleic acid transcripts, proteins, or enzymes) in prokaryotic or eukaryotic cells. For example, CRISPR transcripts can be expressed in bacterial cells such as *Escherichia coli*, insect cells (using baculovirus expression vectors), yeast cells, or mammalian cells. Suitable host cells are discussed further in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

[0119] Vectors may be introduced and propagated in a prokaryote. In some embodiments, a prokaryote is used to amplify copies of a vector to be introduced into a eukaryotic cell or as an intermediate vector in the production of a vector to be introduced into a eukaryotic cell (e.g. amplifying a plasmid as part of a viral vector packaging system). In some embodiments, a prokaryote is used to amplify copies of a vector and express one or more nucleic acids, such as to provide a source of one or more proteins for delivery to a host cell or host organism. Expression of proteins in prokaryotes is most often carried out in *Escherichia coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, such as to the amino terminus of the recombinant protein. Such fusion vectors may serve one or more purposes, such as: (i) to increase expression of recombinant protein; (ii) to increase the solubility of the recombinant protein; and (iii) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Example fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson, 1988. Gene 67: 31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) that fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

[0120] Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amrann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990) 60-89).

[0121] In some embodiments, a vector is a yeast expression vector. Examples of vectors for expression in yeast *Saccharomyces cerivisae* include pYepSec1 (Baldari, et al., 1987. *EMBO J.* 6: 229-234), pMFa (Kuijan and Herskowitz, 1982. *Cell* 30: 933-943), pJRY88 (Schultz et al., 1987. *Gene* 54: 113-123), pYES2 (Invitrogen Corporation, San Diego, Calif.), and picZ (InVitrogen Corp, San Diego, Calif.).

[0122] In some embodiments, a vector drives protein expression in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., SF9 cells) include the pAc series (Smith, et al., 1983. *Mol. Cell. Biol.* 3: 2156-2165) and the pVL series (Lucklow and Summers, 1989. Virology 170: 31-39).

[0123] In some embodiments, a vector is capable of driving expression of one or more sequences in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, 1987. *Nature* 329: 840) and pMT2PC (Kaufman, et al., 1987. *EMBO J.* 6: 187-195). When used in mammalian cells, the expression vector's control functions are typically provided by one or more regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus, simian virus 40, and others disclosed herein and known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells see, e.g., Chapters 16 and 17 of Sambrook, et al., MOLECULAR CLONING: A LABORATORY MANUAL. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

[0124] In some embodiments, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert, et al., 1987. *Genes Dev.* 1: 268-277), lymphoid-specific promoters (Calame and Eaton, 1988. *Adv. Immunol.* 43: 235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989. *EMBO J.* 8: 729-733) and immunoglobulins (Baneiji, et al., 1983. Cell 33: 729-740; Queen and Baltimore, 1983. *Cell* 33: 741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle, 1989. *Proc. Natl. Acad. Sci. USA* 86: 5473-5477), pancreas-specific promoters (Edlund, et al., 1985. *Science* 230: 912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, e.g., the murine hox promoters (Kessel and Gruss, 1990. *Science* 249: 374-379) and the α-fetoprotein promoter (Campes and Tilghman, 1989. *Genes Dev.* 3: 537-546).

[0125] In some embodiments, a regulatory element is operably linked to one or more elements of a CRISPR system so as to drive expression of the one or more elements of the CRISPR system. In general, CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats), also known as SPIDRs (SPacer Interspersed Direct Repeats), constitute a family of DNA loci that are usually specific to a particular bacterial species. The CRISPR locus comprises a distinct class of interspersed short sequence repeats (SSRs) that were recognized in *E. coli* (Ishino et al., J. Bacteriol., 169:5429-5433 [1987]; and Nakata et al., J. Bacteriol., 171:3553-3556 [1989]), and associated genes. Similar interspersed SSRs have been identified in *Haloferax mediterranei, Streptococcus pyogenes, Anabaena*, and *Mycobacterium tuberculosis* (See, Groenen et al., Mol. Microbiol., 10:1057-1065 [1993]; Hoe et al., Emerg. Infect. Dis., 5:254-263 [1999]; Masepohl et al., Biochim. Biophys. Acta 1307:26-30 [1996]; and Mojica et al., Mol. Microbiol., 17:85-93 [1995]). The CRISPR loci typically differ from other SSRs by the structure of the repeats, which have been termed short regularly spaced repeats (SRSRs) (Janssen et al., OMICS J. Integ. Biol., 6:23-33 [2002]; and Mojica et al., Mol. Microbiol., 36:244-246 [2000]). In general, the repeats are short elements that occur in clusters that are regularly spaced by unique intervening sequences with a substantially constant length (Mojica et al.,

[2000], supra). Although the repeat sequences are highly conserved between strains, the number of interspersed repeats and the sequences of the spacer regions typically differ from strain to strain (van Embden et al., J. Bacteriol., 182:2393-2401 [2000]). CRISPR loci have been identified in more than 40 prokaryotes (See e.g., Jansen et al., Mol. Microbiol., 43:1565-1575 [2002]; and Mojica et al.,

[0126] including, but not limited to *Aeropyrum, Pyrobaculum, Sulfolobus, Archaeoglobus, Halocarcula, Methanobacterium, Methanococcus, Methanosarcina, Methanopyrus, Pyrococcus, Picrophilus, Thermoplasma, Corynebacterium, Mycobacterium, Streptomyces, Aquifex, Porphyromonas, Chlorobium, Thermus, Bacillus, Listeria, Staphylococcus, Clostridium, Thermoanaerobacter, Mycoplasma, Fusobacterium, Azarcus, Chromobacterium, Neisseria, Nitrosomonas, Desulfovibrio, Geobacter, Myxococcus, Campylobacter, Wolinella, Acinetobacter, Erwinia, Escherichia, Legionella, Methylococcus, Pasteurella, Photobacterium, Salmonella, Xanthomonas, Yersinia, Treponema*, and *Thermotoga.*

[0127] In general, "CRISPR system" refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), or other sequences and transcripts from a CRISPR locus. In some embodiments, one or more elements of a CRISPR system is derived from a type I, type II, or type III CRISPR system. In some embodiments, one or more elements of a CRISPR system is derived from a particular organism comprising an endogenous CRISPR system, such as *Streptococcus pyogenes*. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. Full complementarity is not necessarily required, provided there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell. In some embodiments, the target sequence may be within an organelle of a eukaryotic cell, for example, mitochondrion or chloroplast. A sequence or template that may be used for recombination into the targeted locus comprising the target sequences is referred to as an "editing template" or "editing polynucleotide" or "editing sequence". In aspects of the invention, an exogenous template polynucleotide may be referred to as an editing template. In an aspect of the invention the recombination is homologous recombination.

[0128] Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (comprising a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. Without wishing to be bound by theory, the tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence. In some embodiments, the tracr sequence has sufficient complementarity to a tracr mate sequence to hybridize and participate in formation of a CRISPR complex. As with the target sequence, it is believed that complete complementarity is not needed, provided there is sufficient to be functional. In some embodiments, the tracr sequence has at least 50%, 60%, 70%, 80%, 90%, 95% or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. In some embodiments, one or more vectors driving expression of one or more elements of a CRISPR system are introduced into a host cell such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. The coding sequence of one element may be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction. In some embodiments, a single promoter drives expression of a transcript encoding a CRISPR enzyme and one or more of the guide sequence, tracr mate sequence (optionally operably linked to the guide sequence), and a tracr sequence embedded within one or more intron sequences (e.g. each in a different intron, two or more in at least one intron, or all in a single intron). In some embodiments, the CRISPR enzyme, guide sequence, tracr mate sequence, and tracr sequence are operably linked to and expressed from the same promoter.

[0129] In some embodiments, a vector comprises one or more insertion sites, such as a restriction endonuclease recognition sequence (also referred to as a "cloning site"). In some embodiments, one or more insertion sites (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more insertion sites) are located upstream and/or downstream of one or more sequence elements of one or more vectors. In some embodiments, a vector comprises an insertion site upstream of a tracr mate sequence, and optionally downstream of a regulatory element operably linked to the tracr mate sequence, such that following insertion of a guide sequence into the insertion site and upon expression the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell. In some embodiments, a vector comprises two or more insertion sites, each insertion site being located between two tracr mate sequences so as to allow insertion of a guide sequence at each site. In such an arrangement, the two or more guide sequences may comprise two or more copies of a single guide sequence, two or more different guide sequences, or combinations of these. When multiple different

guide sequences are used, a single expression construct may be used to target CRISPR activity to multiple different, corresponding target sequences within a cell. For example, a single vector may comprise about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or more guide sequences. In some embodiments, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more such guide-sequence-containing vectors may be provided, and optionally delivered to a cell.

[0130] In some embodiments, a vector comprises a regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, such as a Cas protein. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologs thereof, or modified versions thereof. These enzymes are known; for example, the amino acid sequence of *S. pyogenes* Cas9 protein may be found in the SwissProt database under accession number Q99ZW2. In some embodiments, the unmodified CRISPR enzyme has DNA cleavage activity, such as Cas9. In some embodiments the CRISPR enzyme is Cas9, and may be Cas9 from *S. pyogenes* or *S. pneumoniae*. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence. In some embodiments, a vector encodes a CRISPR enzyme that is mutated to with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D 10A) in the RuvC I catalytic domain of Cas9 from *S. pyogenes* converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). Other examples of mutations that render Cas9 a nickase include, without limitation, H840A, N854A, and N863A. In some embodiments, a Cas9 nickase may be used in combination with guide sequenc(es), e.g., two guide sequences, which target respectively sense and antisense strands of the DNA target. This combination allows both strands to be nicked and used to induce NHEJ. Applicants have demonstrated (data not shown) the efficacy of two nickase targets (i.e., sgRNAs targeted at the same location but to different strands of DNA) in inducing mutagenic NHEJ. A single nickase (Cas9-D10A with a single sgRNA) is unable to induce NHEJ and create indels but Applicants have shown that double nickase (Cas9-D10A and two sgRNAs targeted to different strands at the same location) can do so in human embryonic stem cells (hESCs). The efficiency is about 50% of nuclease (i.e., regular Cas9 without D10 mutation) in hESCs.

[0131] As a further example, two or more catalytic domains of Cas9 (RuvC I, RuvC II, and RuvC III) may be mutated to produce a mutated Cas9 substantially lacking all DNA cleavage activity. In some embodiments, a D10A mutation is combined with one or more of H840A, N854A, or N863A mutations to produce a Cas9 enzyme substantially lacking all DNA cleavage activity. In some embodiments, a CRISPR enzyme is considered to substantially lack all DNA cleavage activity when the DNA cleavage activity of the mutated enzyme is less than about 25%, 10%, 5%, 1%, 0.1%, 0.01%, or lower with respect to its non-mutated form. Other mutations may be useful; where the Cas9 or other CRISPR enzyme is from a species other than *S. pyogenes*, mutations in corresponding amino acids may be made to achieve similar effects.

[0132] In some embodiments, an enzyme coding sequence encoding a CRISPR enzyme is codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a mammal, including but not limited to human, mouse, rat, rabbit, dog, or non-human primate. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the "Codon Usage Database", and these tables can be adapted in a number of ways. See Nakamura, Y., et al. "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" Nucl. Acids Res. 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, Pa.), are also available. In some embodiments, one or more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a CRISPR enzyme correspond to the most frequently used codon for a particular amino acid.

[0133] In some embodiments, a vector encodes a CRISPR enzyme comprising one or more nuclear localization sequences (NLSs), such as about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs. In some embodiments, the CRISPR enzyme comprises about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the amino-terminus, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the carboxy-terminus, or a combination of these (e.g. one or more NLS at the amino-terminus and one or more NLS at the carboxy terminus). When more than one NLS is present, each may be selected independently of the others, such that a single NLS may be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies. In a preferred embodiment of the invention, the CRISPR enzyme comprises at most 6 NLSs. In some embodiments, an NLS is considered near the N- or C-terminus when the nearest amino acid of the NLS is within about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, or more amino acids along the polypeptide chain from the N- or C-terminus. Typically, an NLS consists of one or more short sequences of positively charged lysines or arginines exposed on the protein surface, but other types of NLS are known.

Non-limiting examples of NLSs include an NLS sequence derived from: the NLS of the SV40 virus large T-antigen, having the amino acid sequence PKKKRKV (SEQ ID NO: 1); the NLS from nucleoplasmin (e.g. the nucleoplasmin bipartite NLS with the sequence KRPAATKKAGQAKKKK (SEQ ID NO: 2)); the c-myc NLS having the amino acid sequence PAAKRVKLD (SEQ ID NO: 3) or RQRRNELKRSP (SEQ ID NO: 4); the hRNPA1 M9 NLS having the sequence NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY (SEQ ID NO: 5); the sequence RMRIZFKNKGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV (SEQ ID NO: 6) of the IBB domain from importin-alpha; the sequences VSRKRPRP (SEQ ID NO: 7) and PPKKARED (SEQ ID NO: 8) of the myoma T protein; the sequence PQPKKKPL (SEQ ID NO: 9) of human p53; the sequence SALIKKKKKMAP (SEQ ID NO: 10) of mouse c-abl IV; the sequences DRLRR (SEQ ID NO: 11) and PKQKKRK (SEQ ID NO: 12) of the influenza virus NS1; the sequence RKLKKKIKKL (SEQ ID NO: 13) of the Hepatitis virus delta antigen; the sequence REKKKFLKRR (SEQ ID NO: 14) of the mouse Mx1 protein; the sequence KRKGDEVDGVDEVAKKKSKK (SEQ ID NO: 15) of the human poly(ADP-ribose) polymerase; and the sequence RKCLQAGMNLEARKTKK (SEQ ID NO: 16) of the steroid hormone receptors (human) glucocorticoid.

[0134] In general, the one or more NLSs are of sufficient strength to drive accumulation of the CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In general, strength of nuclear localization activity may derive from the number of NLSs in the CRISPR enzyme, the particular NLS(s) used, or a combination of these factors. Detection of accumulation in the nucleus may be performed by any suitable technique. For example, a detectable marker may be fused to the CRISPR enzyme, such that location within a cell may be visualized, such as in combination with a means for detecting the location of the nucleus (e.g. a stain specific for the nucleus such as DAPI). Examples of detectable markers include fluorescent proteins (such as Green fluorescent proteins, or GFP; RFP; CFP), and epitope tags (HA tag, flag tag, SNAP tag). Cell nuclei may also be isolated from cells, the contents of which may then be analyzed by any suitable process for detecting protein, such as immunohistochemistry, Western blot, or enzyme activity assay. Accumulation in the nucleus may also be determined indirectly, such as by an assay for the effect of CRISPR complex formation (e.g. assay for DNA cleavage or mutation at the target sequence, or assay for altered gene expression activity affected by CRISPR complex formation and/or CRISPR enzyme activity), as compared to a control no exposed to the CRISPR enzyme or complex, or exposed to a CRISPR enzyme lacking the one or more NLSs.

[0135] In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g. the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies, ELAND (Illumina, San Diego, Calif.), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). In some embodiments, a guide sequence is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guide sequence is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. The ability of a guide sequence to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide sequence to be tested and a control guide sequence different from the test guide sequence, and comparing binding or rate of cleavage at the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art.

[0136] A guide sequence may be selected to target any target sequence. In some embodiments, the target sequence is a sequence within a genome of a cell. Exemplary target sequences include those that are unique in the target genome. For example, for the *S. pyogenes* Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNXGG where NNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMNNNNNNNNNNXGG where NNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. For the *S. thermophilus* CRISPR1 Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNXXAGAAW (SEQ ID NO: 17) where NNNNNNNNNNXXAGAAW (SEQ ID NO: 18) (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. thermophilus* CRISPR1 Cas9 target site of the form MMMMMMMMNNNNNNNNNNXXAGAAW (SEQ ID NO: 19) where NNNNNNNNNNXXAGAAW (SEQ ID NO: 20) (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. For the *S. pyogenes* Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNXGGXG where NNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMNNNNNNNNNNXGGXG where NNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. In each of

these sequences "M" may be A, G, T, or C, and need not be considered in identifying a sequence as unique.

[0137] In some embodiments, a guide sequence is selected to reduce the degree of secondary structure within the guide sequence. Secondary structure may be determined by any suitable polynucleotide folding algorithm. Some programs are based on calculating the minimal Gibbs free energy. An example of one such algorithm is mFold, as described by Zuker and Stiegler (Nucleic Acids Res. 9 (1981), 133-148). Another example folding algorithm is the online webserver RNAfold, developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g. A. R. Gruber et al., 2008, Cell 106(1): 23-24; and PA Can and GM Church, 2009, *Nature Biotechnology* 27(12): 1151-62). Further algorithms may be found in U.S. application Ser. No. TBA (attorney docket 44790.11.2022; Broad Reference BI-2013/004A); incorporated herein by reference.

[0138] In general, a tracr mate sequence includes any sequence that has sufficient complementarity with a tracr sequence to promote one or more of: (1) excision of a guide sequence flanked by tracr mate sequences in a cell containing the corresponding tracr sequence; and (2) formation of a CRISPR complex at a target sequence, wherein the CRISPR complex comprises the tracr mate sequence hybridized to the tracr sequence. In general, degree of complementarity is with reference to the optimal alignment of the tracr mate sequence and tracr sequence, along the length of the shorter of the two sequences. Optimal alignment may be determined by any suitable alignment algorithm, and may further account for secondary structures, such as self-complementarity within either the tracr sequence or tracr mate sequence. In some embodiments, the degree of complementarity between the tracr sequence and tracr mate sequence along the length of the shorter of the two when optimally aligned is about or more than about 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97.5%, 99%, or higher. Example illustrations of optimal alignment between a tracr sequence and a tracr mate sequence are provided in FIGS. **12**B and **13**B. In some embodiments, the tracr sequence is about or more than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or more nucleotides in length. In some embodiments, the tracr sequence and tracr mate sequence are contained within a single transcript, such that hybridization between the two produces a transcript having a secondary structure, such as a hairpin. Preferred loop forming sequences for use in hairpin structures are four nucleotides in length, and most preferably have the sequence GAAA. However, longer or shorter loop sequences may be used, as may alternative sequences. The sequences preferably include a nucleotide triplet (for example, AAA), and an additional nucleotide (for example C or G). Examples of loop forming sequences include CAAA and AAAG. In an embodiment of the invention, the transcript or transcribed polynucleotide sequence has at least two or more hairpins. In preferred embodiments, the transcript has two, three, four or five hairpins. In a further embodiment of the invention, the transcript has at most five hairpins. In some embodiments, the single transcript further includes a transcription termination sequence; preferably this is a polyT sequence, for example six T nucleotides. An example illustration of such a hairpin structure is provided in the lower portion of FIG. **13**B, where the portion of the sequence 5' of the final "N" and upstream of the loop corresponds to the tracr mate sequence, and the portion of the sequence 3' of the loop corresponds to the tracr sequence. Further non-limiting examples of single polynucleotides comprising a guide sequence, a tracr mate sequence, and a tracr sequence are as follows (listed 5' to 3'), where "N" represents a base of a guide sequence, the first block of lower case letters represent the tracr mate sequence, and the second block of lower case letters represent the tracr sequence, and the final poly-T sequence represents the transcription terminator: (1) NNNNNNNNNNNNNNNNNNgtttttg-tactctcaagatttaGAAAtaaatcttgcagaagctacaaagataaggctt cat-gccgaaatcaacaccctgtcatttttatggcagggtgttttcgttatttaaTTTTTT (SEQ ID NO: 21); (2) NNNNNNNNNNNNNNNNNNgtttttg-tactctcaGAAAtgcagaagctacaaagataaggcttcatgccgaaatca acaccctgtcatttttatggcagggtgttttcgttatttaaTTTTTT (SEQ ID NO: 22); (3) NNNNNNNNNNNNNNNNNN gatttgtactctca-GAAAtgcagaagctacaaagataaggcttcatgccgaaatca acaccctgt-cattttatggcagggtgtTTTTTT (SEQ ID NO: 23); (4) NNNNNNNNNNNNNNNNNNNNgtttta-gagctaGAAAtagcaagttaaaataaggctagtccgttatcaacttgaaaa agtggcaccgagtcggtgcTTTTTT (SEQ ID NO: 24); (5) gtttta-gagctaGAAATAGcaagttaaaataaggctagtccgttatcaacttgaa aaagtgTTTTTTT (SEQ ID NO: 25); and (6) NNNNNNNNNNNNNNNNNNNNgtttta-gagctagAAATAGcaagttaaaataaggctagtccgttatcaTTTTT TTT (SEQ ID NO: 26). In some embodiments, sequences (1) to (3) are used in combination with Cas9 from *S. thermophilus* CRISPR1. In some embodiments, sequences (4) to (6) are used in combination with Cas9 from *S. pyogenes*. In some embodiments, the tracr sequence is a separate transcript from a transcript comprising the tracr mate sequence (such as illustrated in the top portion of FIG. **13**B).

[0139] In some embodiments, a recombination template is also provided. A recombination template may be a component of another vector as described herein, contained in a separate vector, or provided as a separate polynucleotide. In some embodiments, a recombination template is designed to serve as a template in homologous recombination, such as within or near a target sequence nicked or cleaved by a CRISPR enzyme as a part of a CRISPR complex. A template polynucleotide may be of any suitable length, such as about or more than about 10, 15, 20, 25, 50, 75, 100, 150, 200, 500, 1000, or more nucleotides in length. In some embodiments, the template polynucleotide is complementary to a portion of a polynucleotide comprising the target sequence. When optimally aligned, a template polynucleotide might overlap with one or more nucleotides of a target sequences (e.g. about or more than about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 or more nucleotides). In some embodiments, when a template sequence and a polynucleotide comprising a target sequence are optimally aligned, the nearest nucleotide of the template polynucleotide is within about 1, 5, 10, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 1000, 5000, 10000, or more nucleotides from the target sequence.

[0140] In some embodiments, the CRISPR enzyme is part of a fusion protein comprising one or more heterologous protein domains (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more domains in addition to the CRISPR enzyme). A CRISPR enzyme fusion protein may comprise any additional protein sequence, and optionally a linker sequence between any two domains. Examples of protein domains that may be fused to a CRISPR enzyme include, without limitation, epitope tags, reporter gene sequences, and protein domains having one or more of the following activi-

ties: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. Non-limiting examples of epitope tags include histidine (His) tags, V5 tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Examples of reporter genes include, but are not limited to, glutathione-S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) beta-galactosidase, beta-glucuronidase, luciferase, green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and autofluorescent proteins including blue fluorescent protein (BFP). A CRISPR enzyme may be fused to a gene sequence encoding a protein or a fragment of a protein that bind DNA molecules or bind other cellular molecules, including but not limited to maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. Additional domains that may form part of a fusion protein comprising a CRISPR enzyme are described in US20110059502, incorporated herein by reference. In some embodiments, a tagged CRISPR enzyme is used to identify the location of a target sequence.

[0141] In some aspects, the invention provides methods comprising delivering one or more polynucleotides, such as or one or more vectors as described herein, one or more transcripts thereof, and/or one or proteins transcribed therefrom, to a host cell. In some aspects, the invention further provides cells produced by such methods, and organisms (such as animals, plants, or fungi) comprising or produced from such cells. In some embodiments, a CRISPR enzyme in combination with (and optionally complexed with) a guide sequence is delivered to a cell. Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids in mammalian cells or target tissues. Such methods can be used to administer nucleic acids encoding components of a CRISPR system to cells in culture, or in a host organism. Non-viral vector delivery systems include DNA plasmids, RNA (e.g. a transcript of a vector described herein), naked nucleic acid, and nucleic acid complexed with a delivery vehicle, such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, Science 256:808-813 (1992); Nabel & Feigner, TIBTECH 11:211-217 (1993); Mitani & Caskey, TIBTECH 11:162-166 (1993); Dillon, TIBTECH 11:167-175 (1993); Miller, Nature 357:455-460 (1992); Van Brunt, Biotechnology 6(10):1149-1154 (1988); Vigne, Restorative Neurology and Neuroscience 8:35-36 (1995); Kremer & Perricaudet, British Medical Bulletin 51(1):31-44 (1995); Haddada et al., in Current Topics in Microbiology and Immunology, Doerfler and Bohm (eds) (1995); and Yu et al., Gene Therapy 1:13-26 (1994).

[0142] Methods of non-viral delivery of nucleic acids include lipofection, nucleofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in e.g., U.S. Pat. Nos. 5,049,386, 4,946,787; and 4,897,355) and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Feigner, WO 91/17424; WO 91/16024. Delivery can be to cells (e.g. in vitro or ex vivo administration) or target tissues (e.g. in vivo administration).

[0143] The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (see, e.g., Crystal, Science 270:404-410 (1995); Blaese et al., Cancer Gene Ther. 2:291-297 (1995); Behr et al., Bioconjugate Chem. 5:382-389 (1994); Remy et al., Bioconjugate Chem. 5:647-654 (1994); Gao et al., Gene Therapy 2:710-722 (1995); Ahmad et al., Cancer Res. 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485, 054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

[0144] The use of RNA or DNA viral based systems for the delivery of nucleic acids takes advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (in vivo) or they can be used to treat cells in vitro, and the modified cells may optionally be administered to patients (ex vivo). Conventional viral based systems could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

[0145] The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are comprised of cis-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof (see, e.g., Buchscher et al., J. Virol. 66:2731-2739 (1992); Johann et al., J. Virol. 66:1635-1640 (1992); Sommnerfelt et al., Virol. 176:58-59 (1990); Wilson et al., J. Virol. 63:2374-2378 (1989); Miller et al., J. Virol. 65:2220-2224 (1991); PCT/US94/05700). In applications where transient expression is preferred, adenoviral based systems may be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors may also be used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and for in vivo and ex vivo gene therapy procedures (see, e.g., West et al., Virology 160:38-47 (1987); U.S. Pat. No. 4,797,368; WO 93/24641; Kotin, Human Gene Therapy 5:793-801 (1994); Muzyczka, J. Clin. Invest. 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin et al., Mol. Cell. Biol. 5:3251-3260 (1985); Tratschin, et al., Mol.

Cell. Biol. 4:2072-2081 (1984); Hermonat & Muzyczka, PNAS 81:6466-6470 (1984); and Samulski et al., J. Virol. 63:03822-3828 (1989).

[0146] Packaging cells are typically used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by producing a cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the polynucleotide(s) to be expressed. The missing viral functions are typically supplied in trans by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely rep and cap, but lacking ITR sequences. The cell line may also be infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV. Additional methods for the delivery of nucleic acids to cells are known to those skilled in the art. See, for example, US20030087817, incorporated herein by reference.

[0147] In some embodiments, a host cell is transiently or non-transiently transfected with one or more vectors described herein. In some embodiments, a cell is transfected as it naturally occurs in a subject. In some embodiments, a cell that is transfected is taken from a subject. In some embodiments, the cell is derived from cells taken from a subject, such as a cell line. A wide variety of cell lines for tissue culture are known in the art. Examples of cell lines include, but are not limited to, C8161, CCRF-CEM, MOLT, mIMCD-3, NHDF, HeLa-S3, Huh1, Huh4, Huh7, HUVEC, HASMC, HEKn, HEKa, MiaPaCell, Pane1, PC-3, TF1, CTLL-2, C1R, Rat6, CV1, RPTE, A10, T24, J82, A375, ARH-77, Calu1, SW480, SW620, SKOV3, SK-UT, CaCo2, P388D1, SEM-K2, WEHI-231, HB56, TIB55, Jurkat, J45.01, LRMB, Bcl-1, BC-3, IC21, DLD2, Raw264.7, NRK, NRK-52E, MRCS, MEF, Hep G2, HeLa B, HeLa T4, COS, COS-1, COS-6, COS-M6A, BS-C-1 monkey kidney epithelial, BALB/3T3 mouse embryo fibroblast, 3T3 Swiss, 3T3-L1, 132-d5 human fetal fibroblasts; 10.1 mouse fibroblasts, 293-T, 3T3, 721, 9L, A2780, A2780ADR, A2780cis, A172, A20, A253, A431, A-549, ALC, B16, B35, BCP-1 cells, BEAS-2B, bEnd.3, BHK-21, BR 293, BxPC3, C3H-10T1/2, C6/36, Cal-27, CHO, CHO-7, CHO-IR, CHO-K1, CHO-K2, CHO-T, CHO Dhfr −/−, COR-L23, COR-L23/CPR, COR-L23/5010, COR-L23/R23, COS-7, COV-434, CML T1, CMT, CT26, D17, DH82, DU145, DuCaP, EL4, EM2, EM3, EMT6/AR1, EMT6/AR10.0, FM3, H1299, H69, HB54, HB55, HCA2, HEK-293, HeLa, Hepa1c1c7, HL-60, HMEC, HT-29, Jurkat, JY cells, K562 cells, Ku812, KCL22, KG1, KYO1, LNCap, Ma-Mel 1-48, MC-38, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, MDA-MB-435, MDCK II, MDCK II, MOR/0.2R, MONO-MAC 6, MTD-1A, MyEnd, NCI-H69/CPR, NCI-H69/LX10, NCI-H69/LX20, NCI-H69/LX4, NIH-3T3, NALM-1, NW-145, OPCN/OPCT cell lines, Peer, PNT-1A/PNT 2, RenCa, RIN-5F, RMA/RMAS, Saos-2 cells, Sf-9, SkBr3, T2, T-47D, T84, THP1 cell line, U373, U87, U937, VCaP, Vero cells, WM39, WT-49, X63, YAC-1, YAR, and transgenic varieties thereof. Cell lines are available from a variety of sources known to those with skill in the art (see, e.g., the American Type Culture Collection (ATCC) (Manassus, Va.)). In some embodiments, a cell transfected with one or more vectors described herein is used to establish a new cell line comprising one or more vector-derived sequences. In some embodiments, a cell transiently transfected with the components of a CRISPR system as described herein (such as by transient transfection of one or more vectors, or transfection with RNA), and modified through the activity of a CRISPR complex, is used to establish a new cell line comprising cells containing the modification but lacking any other exogenous sequence. In some embodiments, cells transiently or non-transiently transfected with one or more vectors described herein, or cell lines derived from such cells are used in assessing one or more test compounds.

[0148] In some embodiments, one or more vectors described herein are used to produce a non-human transgenic animal or transgenic plant. In some embodiments, the transgenic animal is a mammal, such as a mouse, rat, or rabbit. In certain embodiments, the organism or subject is a plant. In certain embodiments, the organism or subject or plant is algae. Methods for producing transgenic plants and animals are known in the art, and generally begin with a method of cell transfection, such as described herein.

[0149] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

[0150] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

[0151] With recent advances in crop genomics, the ability to use CRISPR-Cas systems to perform efficient and cost effective gene editing and manipulation will allow the rapid selection and comparison of single and multiplexed genetic manipulations to transform such genomes for improved production and enhanced traits. In this regard reference is made to US patents and publications: U.S. Pat. No. 6,603,061—*Agrobacterium*-Mediated Plant Transformation Method; U.S. Pat. No. 7,868,149—Plant Genome Sequences and Uses Thereof and US 2009/0100536—Transgenic Plants with Enhanced Agronomic Traits, all the contents and disclosure of each of which are herein incorporated by reference in their entirety. In the practice of the invention, the contents and disclosure of Morrell et al "Crop genomics:advances and applications" Nat Rev Genet. 2011 Dec. 29; 13(2):85-96 are also herein incorporated by reference in their entirety. In an

advantageous embodiment of the invention, the CRISPR/Cas9 system is used to engineer microalgae (Example 15). Accordingly, reference herein to animal cells may also apply, mutatis mutandis, to plant cells unless otherwise apparent.

[0152] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell, which may be in vivo, ex vivo or in vitro. In some embodiments, the method comprises sampling a cell or population of cells from a human or non-human animal or plant (including micro-algae), and modifying the cell or cells. Culturing may occur at any stage ex vivo. The cell or cells may even be re-introduced into the non-human animal or plant (including micro-algae).

[0153] In plants, pathogens are often host-specific. For example, *Fusarium oxysporum* f. sp. *lycopersici* causes tomato wilt but attacks only tomato, and *F. oxysporum f. dianthii Puccinia graminis* f. sp. *tritici* attacks only wheat. Plants have existing and induced defenses to resist most pathogens. Mutations and recombination events across plant generations lead to genetic variability that gives rise to susceptibility, especially as pathogens reproduce with more frequency than plants. In plants there can be non-host resistance, e.g., the host and pathogen are incompatible. There can also be Horizontal Resistance, e.g., partial resistance against all races of a pathogen, typically controlled by many genes and Vertical Resistance, e.g., complete resistance to some races of a pathogen but not to other races, typically controlled by a few genes. In a Gene-for-Gene level, plants and pathogens evolve together, and the genetic changes in one balance changes in other. Accordingly, using Natural Variability, breeders combine most useful genes for Yield, Quality, Uniformity, Hardiness, Resistance. The sources of resistance genes include native or foreign Varieties, Heirloom Varieties, Wild Plant Relatives, and Induced Mutations, e.g., treating plant material with mutagenic agents. Using the present invention, plant breeders are provided with a new tool to induce mutations. Accordingly, one skilled in the art can analyze the genome of sources of resistance genes, and in Varieties having desired characteristics or traits employ the present invention to induce the rise of resistance genes, with more precision than previous mutagenic agents and hence accelerate and improve plant breeding programs.

[0154] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. In some embodiments, the kit comprises a vector system and instructions for using the kit. In some embodiments, the vector system comprises (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting a guide sequence upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence. Elements may be provide individually or in combinations, and may be provided in any suitable container, such as a vial, a bottle, or a tube. In some embodiments, the kit includes instructions in one or more languages, for example in more than one language.

[0155] In some embodiments, a kit comprises one or more reagents for use in a process utilizing one or more of the elements described herein. Reagents may be provided in any suitable container. For example, a kit may provide one or more reaction or storage buffers. Reagents may be provided in a form that is usable in a particular assay, or in a form that requires addition of one or more other components before use (e.g. in concentrate or lyophilized form). A buffer can be any buffer, including but not limited to a sodium carbonate buffer, a sodium bicarbonate buffer, a borate buffer, a Tris buffer, a MOPS buffer, a HEPES buffer, and combinations thereof. In some embodiments, the buffer is alkaline. In some embodiments, the buffer has a pH from about 7 to about 10. In some embodiments, the kit comprises one or more oligonucleotides corresponding to a guide sequence for insertion into a vector so as to operably link the guide sequence and a regulatory element. In some embodiments, the kit comprises a homologous recombination template polynucleotide.

[0156] In one aspect, the invention provides methods for using one or more elements of a CRISPR system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide. The CRISPR complex of the invention has a wide variety of utility including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in a multiplicity of cell types. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene therapy, drug screening, disease diagnosis, and prognosis. An exemplary CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence.

[0157] The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). Without wishing to be bound by theory, it is believed that the target sequence should be associated with a PAM (protospacer adjacent motif); that is, a short sequence recognized by the CRISPR complex. The precise sequence and length requirements for the PAM differ depending on the CRISPR enzyme used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence) Examples of PAM sequences are given in the examples section below, and the skilled person will be able to identify further PAM sequences for use with a given CRISPR enzyme.

[0158] The target polynucleotide of a CRISPR complex may include a number of disease-associated genes and polynucleotides as well as signaling biochemical pathway-associated genes and polynucleotides as listed in U.S. provisional patent application 61/736,527 and 61/748,427 having Broad reference BI-2011/008/WSGR Docket No. 44063-701.101 and BI-2011/008/WSGR Docket No. 44063-701.102 respectively, both entitled SYSTEMS METHODS AND COMPO-

SITIONS FOR SEQUENCE MANIPULATION filed on Dec. 12, 2012 and Jan. 2, 2013, respectively, the contents of all of which are herein incorporated by reference in their entirety.

[0159] Examples of target polynucleotides include a sequence associated with a signaling biochemical pathway, e.g., a signaling biochemical pathway-associated gene or polynucleotide. Examples of target polynucleotides include a disease associated gene or polynucleotide. A "disease-associated" gene or polynucleotide refers to any gene or polynucleotide which is yielding transcription or translation products at an abnormal level or in an abnormal form in cells derived from a disease-affected tissues compared with tissues or cells of a non disease control. It may be a gene that becomes expressed at an abnormally high level; it may be a gene that becomes expressed at an abnormally low level, where the altered expression correlates with the occurrence and/or progression of the disease. A disease-associated gene also refers to a gene possessing mutation(s) or genetic variation that is directly responsible or is in linkage disequilibrium with a gene(s) that is responsible for the etiology of a disease. The transcribed or translated products may be known or unknown, and may be at a normal or abnormal level.

[0160] Examples of disease-associated genes and polynucleotides are available from McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), available on the World Wide Web.

[0161] Examples of disease-associated genes and polynucleotides are listed in Tables A and B. Disease specific information is available from McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), available on the World Wide Web. Examples of signaling biochemical pathway-associated genes and polynucleotides are listed in Table C.

[0162] Mutations in these genes and pathways can result in production of improper proteins or proteins in improper amounts which affect function. Further examples of genes, diseases and proteins are hereby incorporated by reference from U.S. Provisional application 61/736,527 filed on Dec. 12, 2012 and 61/748,427 filed on Feb. 2, 2013. Such genes, proteins and pathways may be the target polynucleotide of a CRISPR complex.

TABLE A

DISEASE/DISORDERS	GENE(S)
Neoplasia	PTEN; ATM; ATR; EGFR; ERBB2; ERBB3; ERBB4; Notch1; Notch2; Notch3; Notch4; AKT; AKT2; AKT3; HIF; HIF1a; HIF3a; Met; HRG; Bcl2; PPAR alpha; PPAR gamma; WT1 (Wilms Tumor); FGF Receptor Family members (5 members: 1, 2, 3, 4, 5); CDKN2a; APC; RB (retinoblastoma); MEN1; VHL; BRCA1; BRCA2; AR (Androgen Receptor); TSG101; IGF; IGF Receptor; Igf1 (4 variants); Igf2 (3 variants); Igf 1 Receptor; Igf 2 Receptor; Bax; Bcl2; caspases family (9 members: 1, 2, 3, 4, 6, 7, 8, 9, 12); Kras; Apc
Age-related Macular Degeneration	Abcr; Ccl2; Cc2; cp (ceruloplasmin); Timp3; cathepsinD; Vldlr; Ccr2
Schizophrenia	Neuregulin1 (Nrg1); Erb4 (receptor for Neuregulin); Complexin1 (Cplx1); Tph1 Tryptophan hydroxylase; Tph2 Tryptophan hydroxylase 2; Neurexin 1; GSK3; GSK3a; GSK3b
Disorders	5-HTT (Slc6a4); COMT; DRD (Drd1a); SLC6A3; DAOA; DTNBP1; Dao (Dao1)
Trinucleotide Repeat Disorders	HTT (Huntington's Dx); SBMA/SMAX1/AR (Kennedy's Dx); FXN/X25 (Friedrich's Ataxia); ATX3 (Machado-Joseph's Dx); ATXN1 and ATXN2 (spinocerebellar ataxias); DMPK (myotonic dystrophy); Atrophin-1 and Atn1 (DRPLA Dx); CBP (Creb-BP - global instability); VLDLR (Alzheimer's); Atxn7; Atxn10
Fragile X Syndrome	FMR2; FXR1; FXR2; mGLUR5
Secretase Related Disorders	APH-1 (alpha and beta); Presenilin (Psen1); nicastrin (Ncstn); PEN-2
Others	Nos1; Parp1; Nat1; Nat2
Prion - related disorders	Prp
ALS	SOD1; ALS2; STEX; FUS; TARDBP; VEGF (VEGF-a; VEGF-b; VEGF-c)
Drug addiction	Prkce (alcohol); Drd2; Drd4; ABAT (alcohol); GRIA2; Grm5; Grin1; Htr1b; Grin2a; Drd3; Pdyn; Gria1 (alcohol)
Autism	Mecp2; BZRAP1; MDGA2; Sema5A; Neurexin 1; Fragile X (FMR2 (AFF2); FXR1; FXR2; Mglur5)
Alzheimer's Disease	E1; CHIP; UCH; UBB; Tau; LRP; PICALM; Clusterin; PS1; SORL1; CR1; Vldlr; Uba1; Uba3; CHIP28 (Aqp1, Aquaporin 1); Uchl1; Uchl3; APP
Inflammation	IL-10; IL-1 (IL-1a; IL-1b); IL-13; IL-17 (IL-17a (CTLA8); IL-17b; IL-17c; IL-17d; IL-17f); II-23; Cx3cr1; ptpn22; TNFa; NOD2/CARD15 for IBD; IL-6; IL-12 (IL-12a; IL-12b); CTLA4; Cx3cl1
Parkinson's Disease	x-Synuclein; DJ-1; LRRK2; Parkin; PINK1

TABLE B

Blood and coagulation diseases and disorders	Anemia (CDAN1, CDA1, RPS19, DBA, PKLR, PK1, NT5C3, UMPH1, PSN1, RHAG, RH50A, NRAMP2, SPTB, ALAS2, ANH1, ASB, ABCB7, ABC7, ASAT); Bare lymphocyte syndrome (TAPBP, TPSN, TAP2, ABCB3, PSF2, RING11, MHC2TA, C2TA, RFX5, RFXAP, RFX5), Bleeding disorders (TBXA2R, P2RX1, P2X1); Factor H and factor H-like 1 (HF1, CFH, HUS); Factor V and factor VIII (MCFD2); Factor VII deficiency (F7); Factor X deficiency (F10); Factor XI deficiency (F11); Factor XII deficiency (F12, HAF); Factor XIIIA deficiency (F13A1, F13A); Factor XIIIB deficiency (F13B); Fanconi anemia (FANCA, FACA, FA1, FA, FAA, FAAP95, FAAP90, FLJ34064, FANCB, FANCC, FACC, BRCA2, FANCD1, FANCD2, FANCD, FACD, FAD, FANCE, FACE, FANCF, XRCC9, FANCG, BRIP1, BACH1, FANCJ, PHF9, FANCL, FANCM, KIAA1596); Hemophagocytic lymphohistiocytosis disorders (PRF1, HPLH2, UNC13D, MUNC13-4, HPLH3, HLH3, FHL3); Hemophilia A (F8, F8C, HEMA); Hemophilia B (F9, HEMB), Hemorrhagic disorders (PI, ATT, F5); Leukocyde deficiencies and disorders (ITGB2, CD18, LCAMB, LAD, EIF2B1, EIF2BA, EIF2B2, EIF2B3, EIF2B5, LVWM, CACH, CLE, EIF2B4); Sickle cell anemia (HBB); Thalassemia (HBA2, HBB, HBD, LCRB, HBA1).
Cell dysregulation and oncology diseases and disorders	B-cell non-Hodgkin lymphoma (BCL7A, BCL7); Leukemia (TAL1, TCL5, SCL, TAL2, FLT3, NBS1, NBS, ZNFN1A1, IK1, LYF1, HOXD4, HOX4B, BCR, CML, PHL, ALL, ARNT, KRAS2, RASK2, GMPS, AF10, ARHGEF12, LARG, KIAA0382, CALM, CLTH, CEBPA, CEBP, CHIC2, BTL, FLT3, KIT, PBT, LPP, NPM1, NUP214, D9S46E, CAN, CAIN, RUNX1, CBFA2, AML1, WHSC1L1, NSD3, FLT3, AF1Q, NPM1, NUMA1, ZNF145, PLZF, PML, MYL, STAT5B, AF10, CALM, CLTH, ARL11, ARLTS1, P2RX7, P2X7, BCR, CML, PHL, ALL, GRAF, NF1, VRNF, WSS, NFNS, PTPN11, PTP2C, SHP2, NS1, BCL2, CCND1, PRAD1, BCL1, TCRA, GATA1, GF1, ERYF1, NFE1, ABL1, NQO1, DIA4, NMOR1, NUP214, D9S46E, CAN, CAIN).
Inflammation and immune related diseases and disorders	AIDS (KIR3DL1, NKAT3, NKB1, AMB11, KIR3DS1, IFNG, CXCL12, SDF1); Autoimmune lymphoproliferative syndrome (TNFRSF6, APT1, FAS, CD95, ALPS1A); Combined immunodeficiency, (IL2RG, SCIDX1, SCIDX, IMD4); HIV-1 (CCL5, SCYA5, D17S136E, TCP228), HIV susceptibility or infection (IL10, CSIF, CMKBR2, CCR2, CMKBR5, CCCKR5 (CCR5)); Immunodeficiencies (CD3E, CD3G, AICDA, AID, HIGM2, TNFRSF5, CD40, UNG, DGU, HIGM4, TNFSF5, CD40LG, HIGM1, IGM, FOXP3, IPEX, AIID, XPID, PIDX, TNFRSF14B, TACI); Inflammation (IL-10, IL-1 (IL-1a, IL-1b), IL-13, IL-17 (IL-17a (CTLA8), IL-17b, IL-17c, IL-17d, IL-17f), II-23, Cx3cr1, ptpn22, TNFa, NOD2/CARD15 for IBD, IL-6, IL-12 (IL-12a, IL-12b), CTLA4, Cx3cl1); Severe combined immunodeficiencies (SCIDs)(JAK3, JAKL, DCLRE1C, ARTEMIS, SCIDA, RAG1, RAG2, ADA, PTPRC, CD45, LCA, IL7R, CD3D, T3D, IL2RG, SCIDX1, SCIDX, IMD4).
Metabolic, liver, kidney and protein diseases and disorders	Amyloid neuropathy (TTR, PALB); Amyloidosis (APOA1, APP, AAA, CVAP, AD1, GSN, FGA, LYZ, TTR, PALB); Cirrhosis (KRT18, KRT8, CIRH1A, NAIC, TEX292, KIAA1988); Cystic fibrosis (CFTR, ABCC7, CF, MRP7); Glycogen storage diseases (SLC2A2, GLUT2, G6PC, G6PT, G6PT1, GAA, LAMP2, LAMPB, AGL, GDE, GBE1, GYS2, PYGL, PFKM); Hepatic adenoma, 142330 (TCF1, HNF1A, MODY3), Hepatic failure, early onset, and neurologic disorder (SCOD1, SCO1), Hepatic lipase deficiency (LIPC), Hepatoblastoma, cancer and carcinomas (CTNNB1, PDGFRL, PDGRL, PRLTS, AXIN1, AXIN, CTNNB1, TP53, P53, LFS1, IGF2R, MPRI, MET, CASP8, MCH5; Medullary cystic kidney disease (UMOD, HNFJ, FJHN, MCKD2, ADMCKD2); Phenylketonuria (PAH, PKU1, QDPR, DHPR, PTS); Polycystic kidney and hepatic disease (FCYT, PKHD1, ARPKD, PKD1, PKD2, PKD4, PKDTS, PRKCSH, G19P1, PCLD, SEC63).
Muscular/Skeletal diseases and disorders	Becker muscular dystrophy (DMD, BMD, MYF6), Duchenne Muscular Dystrophy (DMD, BMD); Emery-Dreifuss muscular dystrophy (LMNA, LMN1, EMD2, FPLD, CMD1A, HGPS, LGMD1B, LMNA, LMN1, EMD2, FPLD, CMD1A); Facioscapulohumeral muscular dystrophy (FSHMD1A, FSHD1A); Muscular dystrophy (FKRP, MDC1C, LGMD2I, LAMA2, LAMM, LARGE, KIAA0609, MDC1D, FCMD, TTID, MYOT, CAPN3, CANP3, DYSF, LGMD2B, SGCG, LGMD2C, DMDA1, SCG3, SGCA, ADL, DAG2, LGMD2D, DMDA2, SGCB, LGMD2E, SGCD, SGD, LGMD2F, CMD1L, TCAP, LGMD2G, CMD1N, TRIM32, HT2A, LGMD2H, FKRP, MDC1C, LGMD2I, TTN, CMD1G, TMD, LGMD2J, POMT1, CAV3, LGMD1C, SEPN1, SELN, RSMD1, PLEC1, PLTN, EBS1); Osteopetrosis (LRP5, BMND1, LRP7, LR3, OPPG, VBCH2, CLCN7, CLC7, OPTA2, OSTM1, GL, TCIRG1, TIRC7, OC116, OPTB1); Muscular atrophy (VAPB, VAPC, ALS8, SMN1, SMA1, SMA2, SMA3, SMA4, BSCL2, SPG17, GARS, SMAD1, CMT2D, HEXB, IGHMBP2, SMUBP2, CATF1, SMARD1).

TABLE B-continued

Neurological and neuronal diseases and disorders	ALS (SOD1, ALS2, STEX, FUS, TARDBP, VEGF (VEGF-a, VEGF-b, VEGF-c); Alzheimer disease (APP, AAA, CVAP, AD1, APOE, AD2, PSEN2, AD4, STM2, APBB2, FE65L1, NOS3, PLAU, URK, ACE, DCP1, ACE1, MPO, PACIP1, PAXIP1L, PTIP, A2M, BLMH, BMH, PSEN1, AD3); Autism (Mecp2, BZRAP1, MDGA2, Sema5A, Neurexin1, GLO1, MECP2, RTT, PPMX, MRX16, MRX79, NLGN3, NLGN4, KIAA1260, AUTSX2); Fragile X Syndrome (FMR2, FXR1, FXR2, mGLUR5); Huntington's disease and disease like disorders (HD, IT15, PRNP, PRIP, JPH3, JP3, HDL2, TBP, SCA17); Parkinson disease (NR4A2, NURR1, NOT, TINUR, SNCAIP, TBP, SCA17, SNCA, NACP, PARK1, PARK4, DJ1, PARK7, LRRK2, PARK8, PINK1, PARK6, UCHL1, PARK5, SNCA, NACP, PARK1, PARK4, PRKN, PARK2, PDJ, DBH, NDUFV2); Rett syndrome (MECP2, RTT, PPMX, MRX16, MRX79, CDKL5, STK9, MECP2, RTT, PPMX, MRX16, MRX79, x-Synuclein, DJ-1); Schizophrenia (Neuregulin1 (Nrg1), Erb4 (receptor for Neuregulin), Complexin1 (Cplx1), Tph1 Tryptophan hydroxylase, Tph2, Tryptophan hydroxylase 2, Neurexin 1, GSK3, GSK3a, GSK3b, 5-HTT (Slc6a4), COMT, DRD (Drd1a), SLC6A3, DAOA, DTNBP1, Dao (Dao1)); Secretase Related Disorders (APH-1 (alpha and beta), Presenilin (Psen1), nicastrin, (Ncstn), PEN-2, Nos1, Parp1, Nat1, Nat2); Trinucleotide Repeat Disorders (HTT (Huntington's Dx), SBMA/SMAX1/AR (Kennedy's Dx), FXN/X25 (Friedrich's Ataxia), ATX3 (Machado-Joseph's Dx), ATXN1 and ATXN2 (spinocerebellar ataxias), DMPK (myotonic dystrophy), Atrophin-1 and Atn1 (DRPLA Dx), CBP (Creb-BP-global instability), VLDLR (Alzheimer's), Atxn7, Atxn10).
Occular diseases and disorders	Age-related macular degeneration (Abcr, Ccl2, Cc2, cp (ceruloplasmin), Timp3, cathepsinD, Vldlr, Ccr2); Cataract (CRYAA, CRYA1, CRYBB2, CRYB2, PITX3, BFSP2, CP49, CP47, CRYAA, CRYA1, PAX6, AN2, MGDA, CRYBA1, CRYB1, CRYGC, CRYG3, CCL, LIM2, MP19, CRYGD, CRYG4, BFSP2, CP49, CP47, HSF4, CTM, HSF4, CTM, MIP, AQP0, CRYAB, CRYA2, CTPP2, CRYBB1, CRYGD, CRYG4, CRYBB2, CRYB2, CRYGC, CRYG3, CCL, CRYAA, CRYA1, GJA8, CX50, CAE1, GJA3, CX46, CZP3, CAE3, CCM1, CAM, KRIT1); Corneal clouding and dystrophy (APOA1, TGFBI, CSD2, CDGG1, CSD, BIGH3, CDG2, TACSTD2, TROP2, M1S1, VSX1, RINX, PPCD, PPD, KTCN, COL8A2, FECD, PPCD2, PIP5K3, CFD); Cornea plana congenital (KERA, CNA2); Glaucoma (MYOC, TIGR, GLC1A, JOAG, GPOA, OPTN, GLC1E, FIP2, HYPL, NRP, CYP1B1, GLC3A, OPA1, NTG, NPG, CYP1B1, GLC3A); Leber congenital amaurosis (CRB1, RP12, CRX, CORD2, CRD, RPGRIP1, LCA6, CORD9, RPE65, RP20, AIPL1, LCA4, GUCY2D, GUC2D, LCA1, CORD6, RDH12, LCA3); Macular dystrophy (ELOVL4, ADMD, STGD2, STGD3, RDS, RP7, PRPH2, PRPH, AVMD, AOFMD, VMD2).

TABLE C

CELLULAR FUNCTION	GENES
PI3K/AKT Signaling	PRKCE; ITGAM; ITGA5; IRAK1; PRKAA2; EIF2AK2; PTEN; EIF4E; PRKCZ; GRK6; MAPK1; TSC1; PLK1; AKT2; IKBKB; PIK3CA; CDK8; CDKN1B; NFKB2; BCL2; PIK3CB; PPP2R1A; MAPK8; BCL2L1; MAPK3; TSC2; ITGA1; KRAS; EIF4EBP1; RELA; PRKCD; NOS3; PRKAA1; MAPK9; CDK2; PPP2CA; PIM1; ITGB7; YWHAZ; ILK; TP53; RAF1; IKBKG; RELB; DYRK1A; CDKN1A; ITGB1; MAP2K2; JAK1; AKT1; JAK2; PIK3R1; CHUK; PDPK1; PPP2R5C; CTNNB1; MAP2K1; NFKB1; PAK3; ITGB3; CCND1; GSK3A; FRAP1; SFN; ITGA2; TTK; CSNK1A1; BRAF; GSK3B; AKT3; FOXO1; SGK; HSP90AA1; RPS6KB1
ERK/MAPK Signaling	PRKCE; ITGAM; ITGA5; HSPB1; IRAK1; PRKAA2; EIF2AK2; RAC1; RAP1A; TLN1; EIF4E; ELK1; GRK6; MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8; CREB1; PRKCI; PTK2; FOS; RPS6KA4; PIK3CB; PPP2R1A; PIK3C3; MAPK8; MAPK3; ITGA1; ETS1; KRAS; MYCN; EIF4EBP1; PPARG; PRKCD; PRKAA1; MAPK9; SRC; CDK2; PPP2CA; PIM1; PIK3C2A; ITGB7; YWHAZ; PPP1CC; KSR1; PXN; RAF1; FYN; DYRK1A; ITGB1; MAP2K2; PAK4; PIK3R1; STAT3; PPP2R5C; MAP2K1; PAK3; ITGB3; ESR1; ITGA2; MYC; TTK; CSNK1A1; CRKL; BRAF; ATF4; PRKCA; SRF; STAT1; SGK

TABLE C-continued

CELLULAR FUNCTION	GENES
Glucocorticoid Receptor Signaling	RAC1; TAF4B; EP300; SMAD2; TRAF6; PCAF; ELK1; MAPK1; SMAD3; AKT2; IKBKB; NCOR2; UBE2I; PIK3CA; CREB1; FOS; HSPA5; NFKB2; BCL2; MAP3K14; STAT5B; PIK3CB; PIK3C3; MAPK8; BCL2L1; MAPK3; TSC22D3; MAPK10; NRIP1; KRAS; MAPK13; RELA; STAT5A; MAPK9; NOS2A; PBX1; NR3C1; PIK3C2A; CDKN1C; TRAF2; SERPINE1; NCOA3; MAPK14; TNF; RAF1; IKBKG; MAP3K7; CREBBP; CDKN1A; MAP2K2; JAK1; IL8; NCOA2; AKT1; JAK2; PIK3R1; CHUK; STAT3; MAP2K1; NFKB1; TGFBR1; ESR1; SMAD4; CEBPB; JUN; AR; AKT3; CCL2; MMP1; STAT1; IL6; HSP90AA1
Axonal Guidance Signaling	PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; ADAM12; IGF1; RAC1; RAP1A; EIF4E; PRKCZ; NRP1; NTRK2; ARHGEF7; SMO; ROCK2; MAPK1; PGF; RAC2; PTPN11; GNAS; AKT2; PIK3CA; ERBB2; PRKCI; PTK2; CFL1; GNAQ; PIK3CB; CXCL12; PIK3C3; WNT11; PRKD1; GNB2L1; ABL1; MAPK3; ITGA1; KRAS; RHOA; PRKCD; PIK3C2A; ITGB7; GLI2; PXN; VASP; RAF1; FYN; ITGB1; MAP2K2; PAK4; ADAM17; AKT1; PIK3R1; GLI1; WNT5A; ADAM10; MAP2K1; PAK3; ITGB3; CDC42; VEGFA; ITGA2; EPHA8; CRKL; RND1; GSK3B; AKT3; PRKCA
Ephrin Receptor Signaling	PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; IRAK1; PRKAA2; EIF2AK2; RAC1; RAP1A; GRK6; ROCK2; MAPK1; PGF; RAC2; PTPN11; GNAS; PLK1; AKT2; DOK1; CDK8; CREB1; PTK2; CFL1; GNAQ; MAP3K14; CXCL12; MAPK8; GNB2L1; ABL1; MAPK3; ITGA1; KRAS; RHOA; PRKCD; PRKAA1; MAPK9; SRC; CDK2; PIM1; ITGB7; PXN; RAF1; FYN; DYRK1A; ITGB1; MAP2K2; PAK4; AKT1; JAK2; STAT3; ADAM10; MAP2K1; PAK3; ITGB3; CDC42; VEGFA; ITGA2; EPHA8; TTK; CSNK1A1; CRKL; BRAF; PTPN13; ATF4; AKT3; SGK
Actin Cytoskeleton Signaling	ACTN4; PRKCE; ITGAM; ROCK1; ITGA5; IRAK1; PRKAA2; EIF2AK2; RAC1; INS; ARHGEF7; GRK6; ROCK2; MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8; PTK2; CFL1; PIK3CB; MYH9; DIAPH1; PIK3C3; MAPK8; F2R; MAPK3; SLC9A1; ITGA1; KRAS; RHOA; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; ITGB7; PPP1CC; PXN; VIL2; RAF1; GSN; DYRK1A; ITGB1; MAP2K2; PAK4; PIP5K1A; PIK3R1; MAP2K1; PAK3; ITGB3; CDC42; APC; ITGA2; TTK; CSNK1A1; CRKL; BRAF; VAV3; SGK
Huntington's Disease Signaling	PRKCE; IGF1; EP300; RCOR1; PRKCZ; HDAC4; TGM2; MAPK1; CAPNS1; AKT2; EGFR; NCOR2; SP1; CAPN2; PIK3CA; HDAC5; CREB1; PRKC1; HSPA5; REST; GNAQ; PIK3CB; PIK3C3; MAPK8; IGF1R; PRKD1; GNB2L1; BCL2L1; CAPN1; MAPK3; CASP8; HDAC2; HDAC7A; PRKCD; HDAC11; MAPK9; HDAC9; PIK3C2A; HDAC3; TP53; CASP9; CREBBP; AKT1; PIK3R1; PDPK1; CASP1; APAF1; FRAP1; CASP2; JUN; BAX; ATF4; AKT3; PRKCA; CLTC; SGK; HDAC6; CASP3
Apoptosis Signaling	PRKCE; ROCK1; BID; IRAK1; PRKAA2; EIF2AK2; BAK1; BIRC4; GRK6; MAPK1; CAPNS1; PLK1; AKT2; IKBKB; CAPN2; CDK8; FAS; NFKB2; BCL2; MAP3K14; MAPK8; BCL2L1; CAPN1; MAPK3; CASP8; KRAS; RELA; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; TP53; TNF; RAF1; IKBKG; RELB; CASP9; DYRK1A; MAP2K2; CHUK; APAF1; MAP2K1; NFKB1; PAK3; LMNA; CASP2; BIRC2; TTK; CSNK1A1; BRAF; BAX; PRKCA; SGK; CASP3; BIRC3; PARP1
B Cell Receptor Signaling	RAC1; PTEN; LYN; ELK1; MAPK1; RAC2; PTPN11; AKT2; IKBKB; PIK3CA; CREB1; SYK; NFKB2; CAMK2A; MAP3K14; PIK3CB; PIK3C3; MAPK8; BCL2L1; ABL1; MAPK3; ETS1; KRAS; MAPK13; RELA; PTPN6; MAPK9; EGR1; PIK3C2A; BTK; MAPK14; RAF1; IKBKG; RELB; MAP3K7; MAP2K2; AKT1; PIK3R1; CHUK; MAP2K1; NFKB1; CDC42; GSK3A; FRAP1; BCL6; BCL10; JUN; GSK3B; ATF4; AKT3; VAV3; RPS6KB1
Leukocyte Extravasation Signaling	ACTN4; CD44; PRKCE; ITGAM; ROCK1; CXCR4; CYBA; RAC1; RAP1A; PRKCZ; ROCK2; RAC2; PTPN11; MMP14; PIK3CA; PRKCI; PTK2; PIK3CB; CXCL12; PIK3C3; MAPK8; PRKD1; ABL1; MAPK10; CYBB;

TABLE C-continued

CELLULAR FUNCTION	GENES
	MAPK13; RHOA; PRKCD; MAPK9; SRC; PIK3C2A; BTK; MAPK14; NOX1; PXN; VIL2; VASP; ITGB1; MAP2K2; CTNND1; PIK3R1; CTNNB1; CLDN1; CDC42; F11R; ITK; CRKL; VAV3; CTTN; PRKCA; MMP1; MMP9
Integrin Signaling	ACTN4; ITGAM; ROCK1; ITGA5; RAC1; PTEN; RAP1A; TLN1; ARHGEF7; MAPK1; RAC2; CAPNS1; AKT2; CAPN2; PIK3CA; PTK2; PIK3CB; PIK3C3; MAPK8; CAV1; CAPN1; ABL1; MAPK3; ITGA1; KRAS; RHOA; SRC; PIK3C2A; ITGB7; PPP1CC; ILK; PXN; VASP; RAF1; FYN; ITGB1; MAP2K2; PAK4; AKT1; PIK3R1; TNK2; MAP2K1; PAK3; ITGB3; CDC42; RND3; ITGA2; CRKL; BRAF; GSK3B; AKT3
Acute Phase Response Signaling	IRAK1; SOD2; MYD88; TRAF6; ELK1; MAPK1; PTPN11; AKT2; IKBKB; PIK3CA; FOS; NFKB2; MAP3K14; PIK3CB; MAPK8; RIPK1; MAPK3; IL6ST; KRAS; MAPK13; IL6R; RELA; SOCS1; MAPK9; FTL; NR3C1; TRAF2; SERPINE1; MAPK14; TNF; RAF1; PDK1; IKBKG; RELB; MAP3K7; MAP2K2; AKT1; JAK2; PIK3R1; CHUK; STAT3; MAP2K1; NFKB1; FRAP1; CEBPB; JUN; AKT3; IL1R1; IL6
PTEN Signaling	ITGAM; ITGA5; RAC1; PTEN; PRKCZ; BCL2L11; MAPK1; RAC2; AKT2; EGFR; IKBKB; CBL; PIK3CA; CDKN1B; PTK2; NFKB2; BCL2; PIK3CB; BCL2L1; MAPK3; ITGA1; KRAS; ITGB7; ILK; PDGFRB; INSR; RAF1; IKBKG; CASP9; CDKN1A; ITGB1; MAP2K2; AKT1; PIK3R1; CHUK; PDGFRA; PDPK1; MAP2K1; NFKB1; ITGB3; CDC42; CCND1; GSK3A; ITGA2; GSK3B; AKT3; FOXO1; CASP3; RPS6KB1
p53 Signaling	PTEN; EP300; BBC3; PCAF; FASN; BRCA1; GADD45A; BIRC5; AKT2; PIK3CA; CHEK1; TP53INP1; BCL2; PIK3CB; PIK3C3; MAPK8; THBS1; ATR; BCL2L1; E2F1; PMAIP1; CHEK2; TNFRSF10B; TP73; RB1; HDAC9; CDK2; PIK3C2A; MAPK14; TP53; LRDD; CDKN1A; HIPK2; AKT1; PIK3R1; RRM2B; APAF1; CTNNB1; SIRT1; CCND1; PRKDC; ATM; SFN; CDKN2A; JUN; SNAI2; GSK3B; BAX; AKT3
Aryl Hydrocarbon Receptor Signaling	HSPB1; EP300; FASN; TGM2; RXRA; MAPK1; NQO1; NCOR2; SP1; ARNT; CDKN1B; FOS; CHEK1; SMARCA4; NFKB2; MAPK8; ALDH1A1; ATR; E2F1; MAPK3; NRIP1; CHEK2; RELA; TP73; GSTP1; RB1; SRC; CDK2; AHR; NFE2L2; NCOA3; TP53; TNF; CDKN1A; NCOA2; APAF1; NFKB1; CCND1; ATM; ESR1; CDKN2A; MYC; JUN; ESR2; BAX; IL6; CYP1B1; HSP90AA1
Xenobiotic Metabolism Signaling	PRKCE; EP300; PRKCZ; RXRA; MAPK1; NQO1; NCOR2; PIK3CA; ARNT; PRKCI; NFKB2; CAMK2A; PIK3CB; PPP2R1A; PIK3C3; MAPK8; PRKD1; ALDH1A1; MAPK3; NRIP1; KRAS; MAPK13; PRKCD; GSTP1; MAPK9; NOS2A; ABCB1; AHR; PPP2CA; FTL; NFE2L2; PIK3C2A; PPARGC1A; MAPK14; TNF; RAF1; CREBBP; MAP2K2; PIK3R1; PPP2R5C; MAP2K1; NFKB1; KEAP1; PRKCA; EIF2AK3; IL6; CYP1B1; HSP90AA1
SAPK/JNK Signaling	PRKCE; IRAK1; PRKAA2; EIF2AK2; RAC1; ELK1; GRK6; MAPK1; GADD45A; RAC2; PLK1; AKT2; PIK3CA; FADD; CDK8; PIK3CB; PIK3C3; MAPK8; RIPK1; GNB2L1; IRS1; MAPK3; MAPK10; DAXX; KRAS; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; TRAF2; TP53; LCK; MAP3K7; DYRK1A; MAP2K2; PIK3R1; MAP2K1; PAK3; CDC42; JUN; TTK; CSNK1A1; CRKL; BRAF; SGK
PPAr/RXR Signaling	PRKAA2; EP300; INS; SMAD2; TRAF6; PPARA; FASN; RXRA; MAPK1; SMAD3; GNAS; IKBKB; NCOR2; ABCA1; GNAQ; NFKB2; MAP3K14; STAT5B; MAPK8; IRS1; MAPK3; KRAS; RELA; PRKAA1; PPARGC1A; NCOA3; MAPK14; INSR; RAF1; IKBKG; RELB; MAP3K7; CREBBP; MAP2K2; JAK2; CHUK; MAP2K1; NFKB1; TGFBR1; SMAD4; JUN; IL1R1; PRKCA; IL6; HSP90AA1; ADIPOQ
NF-KB Signaling	IRAK1; EIF2AK2; EP300; INS; MYD88; PRKCZ; TRAF6; TBK1; AKT2; EGFR; IKBKB; PIK3CA; BTRC; NFKB2; MAP3K14; PIK3CB; PIK3C3; MAPK8; RIPK1; HDAC2; KRAS; RELA; PIK3C2A; TRAF2; TLR4; PDGFRB; TNF; INSR; LCK; IKBKG; RELB; MAP3K7; CREBBP; AKT1;

TABLE C-continued

CELLULAR FUNCTION	GENES
	PIK3R1; CHUK; PDGFRA; NFKB1; TLR2; BCL10; GSK3B; AKT3; TNFAIP3; IL1R1
Neuregulin Signaling	ERBB4; PRKCE; ITGAM; ITGA5; PTEN; PRKCZ; ELK1; MAPK1; PTPN11; AKT2; EGFR; ERBB2; PRKCI; CDKN1B; STAT5B; PRKD1; MAPK3; ITGA1; KRAS; PRKCD; STAT5A; SRC; ITGB7; RAF1; ITGB1; MAP2K2; ADAM17; AKT1; PIK3R1; PDPK1; MAP2K1; ITGB3; EREG; FRAP1; PSEN1; ITGA2; MYC; NRG1; CRKL; AKT3; PRKCA; HSP90AA1; RPS6KB1
Wnt & Beta catenin Signaling	CD44; EP300; LRP6; DVL3; CSNK1E; GJA1; SMO; AKT2; PIN1; CDH1; BTRC; GNAQ; MARK2; PPP2R1A; WNT11; SRC; DKK1; PPP2CA; SOX6; SFRP2; ILK; LEF1; SOX9; TP53; MAP3K7; CREBBP; TCF7L2; AKT1; PPP2R5C; WNT5A; LRP5; CTNNB1; TGFBR1; CCND1; GSK3A; DVL1; APC; CDKN2A; MYC; CSNK1A1; GSK3B; AKT3; SOX2
Insulin Receptor Signaling	PTEN; INS; EIF4E; PTPN1; PRKCZ; MAPK1; TSC1; PTPN11; AKT2; CBL; PIK3CA; PRKCI; PIK3CB; PIK3C3; MAPK8; IRS1; MAPK3; TSC2; KRAS; EIF4EBP1; SLC2A4; PIK3C2A; PPP1CC; INSR; RAF1; FYN; MAP2K2; JAK1; AKT1; JAK2; PIK3R1; PDPK1; MAP2K1; GSK3A; FRAP1; CRKL; GSK3B; AKT3; FOXO1; SGK; RPS6KB1
IL-6 Signaling	HSPB1; TRAF6; MAPKAPK2; ELK1; MAPK1; PTPN11; IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK3; MAPK10; IL6ST; KRAS; MAPK13; IL6R; RELA; SOCS1; MAPK9; ABCB1; TRAF2; MAPK14; TNF; RAF1; IKBKG; RELB; MAP3K7; MAP2K2; IL8; JAK2; CHUK; STAT3; MAP2K1; NFKB1; CEBPB; JUN; IL1R1; SRF; IL6
Hepatic Cholestasis	PRKCE; IRAK1; INS; MYD88; PRKCZ; TRAF6; PPARA; RXRA; IKBKB; PRKCI; NFKB2; MAP3K14; MAPK8; PRKD1; MAPK10; RELA; PRKCD; MAPK9; ABCB1; TRAF2; TLR4; TNF; INSR; IKBKG; RELB; MAP3K7; IL8; CHUK; NR1H2; TJP2; NFKB1; ESR1; SREBF1; FGFR4; JUN; IL1R1; PRKCA; IL6
IGF-1 Signaling	IGF1; PRKCZ; ELK1; MAPK1; PTPN11; NEDD4; AKT2; PIK3CA; PRKCI; PTK2; FOS; PIK3CB; PIK3C3; MAPK8; IGF1R; IRS1; MAPK3; IGFBP7; KRAS; PIK3C2A; YWHAZ; PXN; RAF1; CASP9; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; IGFBP2; SFN; JUN; CYR61; AKT3; FOXO1; SRF; CTGF; RPS6KB1
NRF2-mediated Oxidative Stress Response	PRKCE; EP300; SOD2; PRKCZ; MAPK1; SQSTM1; NQO1; PIK3CA; PRKCI; FOS; PIK3CB; PIK3C3; MAPK8; PRKD1; MAPK3; KRAS; PRKCD; GSTP1; MAPK9; FTL; NFE2L2; PIK3C2A; MAPK14; RAF1; MAP3K7; CREBBP; MAP2K2; AKT1; PIK3R1; MAP2K1; PPIB; JUN; KEAP1; GSK3B; ATF4; PRKCA; EIF2AK3; HSP90AA1
Hepatic Fibrosis/Hepatic Stellate Cell Activation	EDN1; IGF1; KDR; FLT1; SMAD2; FGFR1; MET; PGF; SMAD3; EGFR; FAS; CSF1; NFKB2; BCL2; MYH9; IGF1R; IL6R; RELA; TLR4; PDGFRB; TNF; RELB; IL8; PDGFRA; NFKB1; TGFBR1; SMAD4; VEGFA; BAX; IL1R1; CCL2; HGF; MMP1; STAT1; IL6; CTGF; MMP9
PPAR Signaling	EP300; INS; TRAF6; PPARA; RXRA; MAPK1; IKBKB; NCOR2; FOS; NFKB2; MAP3K14; STAT5B; MAPK3; NRIP1; KRAS; PPARG; RELA; STAT5A; TRAF2; PPARGC1A; PDGFRB; TNF; INSR; RAF1; IKBKG; RELB; MAP3K7; CREBBP; MAP2K2; CHUK; PDGFRA; MAP2K1; NFKB1; JUN; IL1R1; HSP90AA1
Fc Epsilon RI Signaling	PRKCE; RAC1; PRKCZ; LYN; MAPK1; RAC2; PTPN11; AKT2; PIK3CA; SYK; PRKCI; PIK3CB; PIK3C3; MAPK8; PRKD1; MAPK3; MAPK10; KRAS; MAPK13; PRKCD; MAPK9; PIK3C2A; BTK; MAPK14; TNF; RAF1; FYN; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; AKT3; VAV3; PRKCA
G-Protein Coupled Receptor Signaling	PRKCE; RAP1A; RGS16; MAPK1; GNAS; AKT2; IKBKB; PIK3CA; CREB1; GNAQ; NFKB2; CAMK2A; PIK3CB; PIK3C3; MAPK3; KRAS; RELA; SRC; PIK3C2A; RAF1; IKBKG; RELB; FYN; MAP2K2; AKT1; PIK3R1; CHUK; PDPK1; STAT3; MAP2K1; NFKB1; BRAF; ATF4; AKT3; PRKCA

TABLE C-continued

CELLULAR FUNCTION	GENES
Inositol Phosphate Metabolism	PRKCE; IRAK1; PRKAA2; EIF2AK2; PTEN; GRK6; MAPK1; PLK1; AKT2; PIK3CA; CDK8; PIK3CB; PIK3C3; MAPK8; MAPK3; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; DYRK1A; MAP2K2; PIP5K1A; PIK3R1; MAP2K1; PAK3; ATM; TTK; CSNK1A1; BRAF; SGK
PDGF Signaling	EIF2AK2; ELK1; ABL2; MAPK1; PIK3CA; FOS; PIK3CB; PIK3C3; MAPK8; CAV1; ABL1; MAPK3; KRAS; SRC; PIK3C2A; PDGFRB; RAF1; MAP2K2; JAK1; JAK2; PIK3R1; PDGFRA; STAT3; SPHK1; MAP2K1; MYC; JUN; CRKL; PRKCA; SRF; STAT1; SPHK2
VEGF Signaling	ACTN4; ROCK1; KDR; FLT1; ROCK2; MAPK1; PGF; AKT2; PIK3CA; ARNT; PTK2; BCL2; PIK3CB; PIK3C3; BCL2L1; MAPK3; KRAS; HIF1A; NOS3; PIK3C2A; PXN; RAF1; MAP2K2; ELAVL1; AKT1; PIK3R1; MAP2K1; SFN; VEGFA; AKT3; FOXO1; PRKCA
Natural Killer Cell Signaling	PRKCE; RAC1; PRKCZ; MAPK1; RAC2; PTPN11; KIR2DL3; AKT2; PIK3CA; SYK; PRKCI; PIK3CB; PIK3C3; PRKD1; MAPK3; KRAS; PRKCD; PTPN6; PIK3C2A; LCK; RAF1; FYN; MAP2K2; PAK4; AKT1; PIK3R1; MAP2K1; PAK3; AKT3; VAV3; PRKCA
Cell Cycle: G1/S Checkpoint Regulation	HDAC4; SMAD3; SUV39H1; HDAC5; CDKN1B; BTRC; ATR; ABL1; E2F1; HDAC2; HDAC7A; RB1; HDAC11; HDAC9; CDK2; E2F2; HDAC3; TP53; CDKN1A; CCND1; E2F4; ATM; RBL2; SMAD4; CDKN2A; MYC; NRG1; GSK3B; RBL1; HDAC6
T Cell Receptor Signaling	RAC1; ELK1; MAPK1; IKBKB; CBL; PIK3CA; FOS; NFKB2; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; RELA; PIK3C2A; BTK; LCK; RAF1; IKBKG; RELB; FYN; MAP2K2; PIK3R1; CHUK; MAP2K1; NFKB1; ITK; BCL10; JUN; VAV3
Death Receptor Signaling	CRADD; HSPB1; BID; BIRC4; TBK1; IKBKB; FADD; FAS; NFKB2; BCL2; MAP3K14; MAPK8; RIPK1; CASP8; DAXX; TNFRSF10B; RELA; TRAF2; TNF; IKBKG; RELB; CASP9; CHUK; APAF1; NFKB1; CASP2; BIRC2; CASP3; BIRC3
FGF Signaling	RAC1; FGFR1; MET; MAPKAPK2; MAPK1; PTPN11; AKT2; PIK3CA; CREB1; PIK3CB; PIK3C3; MAPK8; MAPK3; MAPK13; PTPN6; PIK3C2A; MAPK14; RAF1; AKT1; PIK3R1; STAT3; MAP2K1; FGFR4; CRKL; ATF4; AKT3; PRKCA; HGF
GM-CSF Signaling	LYN; ELK1; MAPK1; PTPN11; AKT2; PIK3CA; CAMK2A; STAT5B; PIK3CB; PIK3C3; GNB2L1; BCL2L1; MAPK3; ETS1; KRAS; RUNX1; PIM1; PIK3C2A; RAF1; MAP2K2; AKT1; JAK2; PIK3R1; STAT3; MAP2K1; CCND1; AKT3; STAT1
Amyotrophic Lateral Sclerosis Signaling	BID; IGF1; RAC1; BIRC4; PGF; CAPNS1; CAPN2; PIK3CA; BCL2; PIK3CB; PIK3C3; BCL2L1; CAPN1; PIK3C2A; TP53; CASP9; PIK3R1; RAB5A; CASP1; APAF1; VEGFA; BIRC2; BAX; AKT3; CASP3; BIRC3
JAK/Stat Signaling	PTPN1; MAPK1; PTPN11; AKT2; PIK3CA; STAT5B; PIK3CB; PIK3C3; MAPK3; KRAS; SOCS1; STAT5A; PTPN6; PIK3C2A; RAF1; CDKN1A; MAP2K2; JAK1; AKT1; JAK2; PIK3R1; STAT3; MAP2K1; FRAP1; AKT3; STAT1
Nicotinate and Nicotinamide Metabolism	PRKCE; IRAK1; PRKAA2; EIF2AK2; GRK6; MAPK1; PLK1; AKT2; CDK8; MAPK8; MAPK3; PRKCD; PRKAA1; PBEF1; MAPK9; CDK2; PIM1; DYRK1A; MAP2K2; MAP2K1; PAK3; NT5E; TTK; CSNK1A1; BRAF; SGK
Chemokine Signaling	CXCR4; ROCK2; MAPK1; PTK2; FOS; CFL1; GNAQ; CAMK2A; CXCL12; MAPK8; MAPK3; KRAS; MAPK13; RHOA; CCR3; SRC; PPP1CC; MAPK14; NOX1; RAF1; MAP2K2; MAP2K1; JUN; CCL2; PRKCA
IL-2 Signaling	ELK1; MAPK1; PTPN11; AKT2; PIK3CA; SYK; FOS; STAT5B; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; SOCS1; STAT5A; PIK3C2A; LCK; RAF1; MAP2K2; JAK1; AKT1; PIK3R1; MAP2K1; JUN; AKT3
Synaptic Long Term Depression	PRKCE; IGF1; PRKCZ; PRDX6; LYN; MAPK1; GNAS; PRKCI; GNAQ; PPP2R1A; IGF1R; PRKD1; MAPK3; KRAS; GRN; PRKCD; NOS3; NOS2A; PPP2CA; YWHAZ; RAF1; MAP2K2; PPP2R5C; MAP2K1; PRKCA

TABLE C-continued

CELLULAR FUNCTION	GENES
Estrogen Receptor Signaling	TAF4B; EP300; CARM1; PCAF; MAPK1; NCOR2; SMARCA4; MAPK3; NRIP1; KRAS; SRC; NR3C1; HDAC3; PPARGC1A; RBM9; NCOA3; RAF1; CREBBP; MAP2K2; NCOA2; MAP2K1; PRKDC; ESR1; ESR2
Protein Ubiquitination Pathway	TRAF6; SMURF1; BIRC4; BRCA1; UCHL1; NEDD4; CBL; UBE2I; BTRC; HSPA5; USP7; USP10; FBXW7; USP9X; STUB1; USP22; B2M; BIRC2; PARK2; USP8; USP1; VHL; HSP90AA1; BIRC3
IL-10 Signaling	TRAF6; CCR1; ELK1; IKBKB; SP1; FOS; NFKB2; MAP3K14; MAPK8; MAPK13; RELA; MAPK14; TNF; IKBKG; RELB; MAP3K7; JAK1; CHUK; STAT3; NFKB1; JUN; IL1R1; IL6
VDR/RXR Activation	PRKCE; EP300; PRKCZ; RXRA; GADD45A; HES1; NCOR2; SP1; PRKCI; CDKN1B; PRKD1; PRKCD; RUNX2; KLF4; YY1; NCOA3; CDKN1A; NCOA2; SPP1; LRP5; CEBPB; FOXO1; PRKCA
TGF-beta Signaling	EP300; SMAD2; SMURF1; MAPK1; SMAD3; SMAD1; FOS; MAPK8; MAPK3; KRAS; MAPK9; RUNX2; SERPINE1; RAF1; MAP3K7; CREBBP; MAP2K2; MAP2K1; TGFBR1; SMAD4; JUN; SMAD5
Toll-like Receptor Signaling	IRAK1; EIF2AK2; MYD88; TRAF6; PPARA; ELK1; IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK13; RELA; TLR4; MAPK14; IKBKG; RELB; MAP3K7; CHUK; NFKB1; TLR2; JUN
p38 MAPK Signaling	HSPB1; IRAK1; TRAF6; MAPKAPK2; ELK1; FADD; FAS; CREB1; DDIT3; RPS6KA4; DAXX; MAPK13; TRAF2; MAPK14; TNF; MAP3K7; TGFBR1; MYC; ATF4; IL1R1; SRF; STAT1
Neurotrophin/TRK Signaling	NTRK2; MAPK1; PTPN11; PIK3CA; CREB1; FOS; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; PIK3C2A; RAF1; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; CDC42; JUN; ATF4
FXR/RXR Activation	INS; PPARA; FASN; RXRA; AKT2; SDC1; MAPK8; APOB; MAPK10; PPARG; MTTP; MAPK9; PPARGC1A; TNF; CREBBP; AKT1; SREBF1; FGFR4; AKT3; FOXO1
Synaptic Long Term Potentiation	PRKCE; RAP1A; EP300; PRKCZ; MAPK1; CREB1; PRKCI; GNAQ; CAMK2A; PRKD1; MAPK3; KRAS; PRKCD; PPP1CC; RAF1; CREBBP; MAP2K2; MAP2K1; ATF4; PRKCA
Calcium Signaling	RAP1A; EP300; HDAC4; MAPK1; HDAC5; CREB1; CAMK2A; MYH9; MAPK3; HDAC2; HDAC7A; HDAC11; HDAC9; HDAC3; CREBBP; CALR; CAMKK2; ATF4; HDAC6
EGF Signaling	ELK1; MAPK1; EGFR; PIK3CA; FOS; PIK3CB; PIK3C3; MAPK8; MAPK3; PIK3C2A; RAF1; JAK1; PIK3R1; STAT3; MAP2K1; JUN; PRKCA; SRF; STAT1
Hypoxia Signaling in the Cardiovascular System	EDN1; PTEN; EP300; NQO1; UBE2I; CREB1; ARNT; HIF1A; SLC2A4; NOS3; TP53; LDHA; AKT1; ATM; VEGFA; JUN; ATF4; VHL; HSP90AA1
LPS/IL-1 Mediated Inhibition of RXR Function	IRAK1; MYD88; TRAF6; PPARA; RXRA; ABCA1; MAPK8; ALDH1A1; GSTP1; MAPK9; ABCB1; TRAF2; TLR4; TNF; MAP3K7; NR1H2; SREBF1; JUN; IL1R1
LXR/RXR Activation	FASN; RXRA; NCOR2; ABCA1; NFKB2; IRF3; RELA; NOS2A; TLR4; TNF; RELB; LDLR; NR1H2; NFKB1; SREBF1; IL1R1; CCL2; IL6; MMP9
Amyloid Processing	PRKCE; CSNK1E; MAPK1; CAPNS1; AKT2; CAPN2; CAPN1; MAPK3; MAPK13; MAPT; MAPK14; AKT1; PSEN1; CSNK1A1; GSK3B; AKT3; APP
IL-4 Signaling	AKT2; PIK3CA; PIK3CB; PIK3C3; IRS1; KRAS; SOCS1; PTPN6; NR3C1; PIK3C2A; JAK1; AKT1; JAK2; PIK3R1; FRAP1; AKT3; RPS6KB1
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	EP300; PCAF; BRCA1; GADD45A; PLK1; BTRC; CHEK1; ATR; CHEK2; YWHAZ; TP53; CDKN1A; PRKDC; ATM; SFN; CDKN2A
Nitric Oxide Signaling in the Cardiovascular System	KDR; FLT1; PGF; AKT2; PIK3CA; PIK3CB; PIK3C3; CAV1; PRKCD; NOS3; PIK3C2A; AKT1; PIK3R1; VEGFA; AKT3; HSP90AA1
Purine Metabolism	NME2; SMARCA4; MYH9; RRM2; ADAR; EIF2AK4; PKM2; ENTPD1; RAD51; RRM2B; TJP2; RAD51C; NT5E; POLD1; NME1

TABLE C-continued

CELLULAR FUNCTION	GENES
cAMP-mediated Signaling	RAP1A; MAPK1; GNAS; CREB1; CAMK2A; MAPK3; SRC; RAF1; MAP2K2; STAT3; MAP2K1; BRAF; ATF4
Mitochondrial Dysfunction	SOD2; MAPK8; CASP8; MAPK10; MAPK9; CASP9; PARK7; PSEN1; PARK2; APP; CASP3
Notch Signaling	HES1; JAG1; NUMB; NOTCH4; ADAM17; NOTCH2; PSEN1; NOTCH3; NOTCH1; DLL4
Endoplasmic Reticulum Stress Pathway	HSPA5; MAPK8; XBP1; TRAF2; ATF6; CASP9; ATF4; EIF2AK3; CASP3
Pyrimidine Metabolism	NME2; AICDA; RRM2; EIF2AK4; ENTPD1; RRM2B; NT5E; POLD1; NME1
Parkinson's Signaling	UCHL1; MAPK8; MAPK13; MAPK14; CASP9; PARK7; PARK2; CASP3
Cardiac & Beta Adrenergic Signaling	GNAS; GNAQ; PPP2R1A; GNB2L1; PPP2CA; PPP1CC; PPP2R5C
Glycolysis/Gluconeogenesis	HK2; GCK; GPI; ALDH1A1; PKM2; LDHA; HK1
Interferon Signaling	IRF1; SOCS1; JAK1; JAK2; IFITM1; STAT1; IFIT3
Sonic Hedgehog Signaling	ARRB2; SMO; GLI2; DYRK1A; GLI1; GSK3B; DYRK1B
Glycerophospholipid Metabolism	PLD1; GRN; GPAM; YWHAZ; SPHK1; SPHK2
Phospholipid Degradation	PRDX6; PLD1; GRN; YWHAZ; SPHK1; SPHK2
Tryptophan Metabolism	SIAH2; PRMT5; NEDD4; ALDH1A1; CYP1B1; SIAH1
Lysine Degradation	SUV39H1; EHMT2; NSD1; SETD7; PPP2R5C
Nucleotide Excision Repair Pathway	ERCC5; ERCC4; XPA; XPC; ERCC1
Starch and Sucrose Metabolism	UCHL1; HK2; GCK; GPI; HK1
Aminosugars Metabolism	NQO1; HK2; GCK; HK1
Arachidonic Acid Metabolism	PRDX6; GRN; YWHAZ; CYP1B1
Circadian Rhythm Signaling	CSNK1E; CREB1; ATF4; NR1D1
Coagulation System	BDKRB1; F2R; SERPINE1; F3
Dopamine Receptor Signaling	PPP2R1A; PPP2CA; PPP1CC; PPP2R5C
Glutathione Metabolism	IDH2; GSTP1; ANPEP; IDH1
Glycerolipid Metabolism	ALDH1A1; GPAM; SPHK1; SPHK2
Linoleic Acid Metabolism	PRDX6; GRN; YWHAZ; CYP1B1
Methionine Metabolism	DNMT1; DNMT3B; AHCY; DNMT3A
Pyruvate Metabolism	GLO1; ALDH1A1; PKM2; LDHA
Arginine and Proline Metabolism	ALDH1A1; NOS3; NOS2A
Eicosanoid Signaling	PRDX6; GRN; YWHAZ
Fructose and Mannose Metabolism	HK2; GCK; HK1
Galactose Metabolism	HK2; GCK; HK1
Stilbene, Coumarine and Lignin Biosynthesis	PRDX6; PRDX1; TYR
Antigen Presentation Pathway	CALR; B2M
Biosynthesis of Steroids	NQO1; DHCR7
Butanoate Metabolism	ALDH1A1; NLGN1
Citrate Cycle	IDH2; IDH1
Fatty Acid Metabolism	ALDH1A1; CYP1B1
Glycerophospholipid Metabolism	PRDX6; CHKA
Histidine Metabolism	PRMT5; ALDH1A1
Inositol Metabolism	ERO1L; APEX1
Metabolism of Xenobiotics by Cytochrome p450	GSTP1; CYP1B1
Methane Metabolism	PRDX6; PRDX1
Phenylalanine Metabolism	PRDX6; PRDX1
Propanoate Metabolism	ALDH1A1; LDHA
Selenoamino Acid Metabolism	PRMT5; AHCY

TABLE C-continued

CELLULAR FUNCTION	GENES
Sphingolipid Metabolism	SPHK1; SPHK2
Aminophosphonate Metabolism	PRMT5
Androgen and Estrogen Metabolism	PRMT5
Ascorbate and Aldarate Metabolism	ALDH1A1
Bile Acid Biosynthesis	ALDH1A1
Cysteine Metabolism	LDHA
Fatty Acid Biosynthesis	FASN
Glutamate Receptor Signaling	GNB2L1
NRF2-mediated Oxidative Stress Response	PRDX1
Pentose Phosphate Pathway	GPI
Pentose and Glucuronate Interconversions	UCHL1
Retinol Metabolism	ALDH1A1
Riboflavin Metabolism	TYR
Tyrosine Metabolism	PRMT5, TYR
Ubiquinone Biosynthesis	PRMT5
Valine, Leucine and Isoleucine Degradation	ALDH1A1
Glycine, Serine and Threonine Metabolism	CHKA
Lysine Degradation	ALDH1A1
Pain/Taste	TRPM5; TRPA1
Pain	TRPM7; TRPC5; TRPC6; TRPC1; Cnr1; cnr2; Grk2; Trpa1; Pomc; Cgrp; Crf; Pka; Era; Nr2b; TRPM5; Prkaca; Prkacb; Prkar1a; Prkar2a
Mitochondrial Function	AIF; CytC; SMAC (Diablo); Aifm-1; Aifm-2
Developmental Neurology	BMP-4; Chordin (Chrd); Noggin (Nog); WNT (Wnt2; Wnt2b; Wnt3a; Wnt4; Wnt5a; Wnt6; Wnt7b; Wnt8b; Wnt9a; Wnt9b; Wnt10a; Wnt10b; Wnt16); beta-catenin; Dkk-1; Frizzled related proteins; Otx-2; Gbx2; FGF-8; Reelin; Dab1; unc-86 (Pou4f1 or Brn3a); Numb; Reln

[0163] Embodiments of the invention also relate to methods and compositions related to knocking out genes, amplifying genes and repairing particular mutations associated with DNA repeat instability and neurological disorders (Robert D. Wells, Tetsuo Ashizawa, Genetic Instabilities and Neurological Diseases, Second Edition, Academic Press, Oct. 13, 2011—Medical). Specific aspects of tandem repeat sequences have been found to be responsible for more than twenty human diseases (New insights into repeat instability: role of RNA•DNA hybrids. McIvor E I, Polak U, Napierala M. RNA Biol. 2010 September-October; 7(5):551-8). The CRISPR-Cas system may be harnessed to correct these defects of genomic instability.

[0164] A further aspect of the invention relates to utilizing the CRISPR-Cas system for correcting defects in the EMP2A and EMP2B genes that have been identified to be associated with Lafora disease. Lafora disease is an autosomal recessive condition which is characterized by progressive myoclonus epilepsy which may start as epileptic seizures in adolescence. A few cases of the disease may be caused by mutations in genes yet to be identified. The disease causes seizures, muscle spasms, difficulty walking, dementia, and eventually death. There is currently no therapy that has proven effective against disease progression. Other genetic abnormalities associated with epilepsy may also be targeted by the CRISPR-Cas system and the underlying genetics is further described in Genetics of Epilepsy and Genetic Epilepsies, edited by Giuliano Avanzini, Jeffrey L. Noebels, Mariani Foundation Paediatric Neurology:20; 2009).

[0165] In yet another aspect of the invention, the CRISPR-Cas system may be used to correct ocular defects that arise from several genetic mutations further described in Genetic Diseases of the Eye, Second Edition, edited by Elias I. Traboulsi, Oxford University Press, 2012.

[0166] Several further aspects of the invention relate to correcting defects associated with a wide range of genetic diseases which are further described on the website of the National Institutes of Health under the topic subsection Genetic Disorders (website at health.nih.gov/topic/GeneticDisorders). The genetic brain diseases may include but are not limited to Adrenoleukodystrophy, Agenesis of the Corpus Callosum, Aicardi Syndrome, Alpers' Disease, Alzheimer's Disease, Barth Syndrome, Batten Disease, CADASIL, Cerebellar Degeneration, Fabry's Disease, Gerstmann-Straussler-Scheinker Disease, Huntington's Disease and other Triplet Repeat Disorders, Leigh's Disease, Lesch-Nyhan Syndrome, Menkes Disease, Mitochondrial Myopathies and NINDS Colpocephaly. These diseases are further described on the website of the National Institutes of Health under the subsection Genetic Brain Disorders.

[0167] In some embodiments, the condition may be neoplasia. In some embodiments, where the condition is neopla-

sia, the genes to be targeted are any of those listed in Table A (in this case PTEN and so forth). In some embodiments, the condition may be Age-related Macular Degeneration. In some embodiments, the condition may be a Schizophrenic Disorder. In some embodiments, the condition may be a Trinucleotide Repeat Disorder. In some embodiments, the condition may be Fragile X Syndrome. In some embodiments, the condition may be a Secretase Related Disorder. In some embodiments, the condition may be a Prion—related disorder. In some embodiments, the condition may be ALS. In some embodiments, the condition may be a drug addiction. In some embodiments, the condition may be Autism. In some embodiments, the condition may be Alzheimer's Disease. In some embodiments, the condition may be inflammation. In some embodiments, the condition may be Parkinson's Disease.

[0168] Examples of proteins associated with Parkinson's disease include but are not limited to α-synuclein, DJ-1, LRRK2, PINK1, Parkin, UCHL1, Synphilin-1, and NURR1.

[0169] Examples of addiction-related proteins may include ABAT for example.

[0170] Examples of inflammation-related proteins may include the monocyte chemoattractant protein-1 (MCP1) encoded by the Ccr2 gene, the C—C chemokine receptor type 5 (CCR5) encoded by the Ccr5 gene, the IgG receptor IIB (FCGR2b, also termed CD32) encoded by the Fcgr2b gene, or the Fc epsilon R1 g (FCER1g) protein encoded by the Fcer1g gene, for example.

[0171] Examples of cardiovascular diseases associated proteins may include IL1B (interleukin 1, beta), XDH (xanthine dehydrogenase), TP53 (tumor protein p53), PTGIS (prostaglandin I2 (prostacyclin) synthase), MB (myoglobin), IL4 (interleukin 4), ANGPT1 (angiopoietin 1), ABCG8 (ATP-binding cassette, sub-family G (WHITE), member 8), or CTSK (cathepsin K), for example.

[0172] Examples of Alzheimer's disease associated proteins may include the very low density lipoprotein receptor protein (VLDLR) encoded by the VLDLR gene, the ubiquitin-like modifier activating enzyme 1 (UBA1) encoded by the UBA1 gene, or the NEDD8-activating enzyme E1 catalytic subunit protein (UBE1C) encoded by the UBA3 gene, for example.

[0173] Examples of proteins associated Autism Spectrum Disorder may include the benzodiazapine receptor (peripheral) associated protein 1 (BZRAP 1) encoded by the BZRAP 1 gene, the AF4/FMR2 family member 2 protein (AFF2) encoded by the AFF2 gene (also termed MFR2), the fragile X mental retardation autosomal homolog 1 protein (FXR1) encoded by the FXR1 gene, or the fragile X mental retardation autosomal homolog 2 protein (FXR2) encoded by the FXR2 gene, for example.

[0174] Examples of proteins associated Macular Degeneration may include the ATP-binding cassette, sub-family A (ABC1) member 4 protein (ABCA4) encoded by the ABCR gene, the apolipoprotein E protein (APOE) encoded by the APOE gene, or the chemokine (C—C motif) L1gand 2 protein (CCL2) encoded by the CCL2 gene, for example.

[0175] Examples of proteins associated Schizophrenia may include NRG1, ErbB4, CPLX1, TPH1, TPH2, NRXN1, GSK3A, BDNF, DISC1, GSK3B, and combinations thereof.

[0176] Examples of proteins involved in tumor suppression may include ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related), EGFR (epidermal growth factor receptor), ERBB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2), ERBB3 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 3), ERBB4 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 4), Notch 1, Notch2, Notch 3, or Notch 4, for example.

[0177] Examples of proteins associated with a secretase disorder may include PSENEN (presenilin enhancer 2 homolog (*C. elegans*)), CTSB (cathepsin B), PSEN1 (presenilin 1), APP (amyloid beta (A4) precursor protein), APH1B (anterior pharynx defective 1 homolog B (*C. elegans*)), PSEN2 (presenilin 2 (Alzheimer disease 4)), or BACE1 (beta-site APP-cleaving enzyme 1), for example.

[0178] Examples of proteins associated with Amyotrophic Lateral Sclerosis may include SOD1 (superoxide dismutase 1), ALS2 (amyotrophic lateral sclerosis 2), FUS (fused in sarcoma), TARDBP (TAR DNA binding protein), VAGFA (vascular endothelial growth factor A), VAGFB (vascular endothelial growth factor B), and VAGFC (vascular endothelial growth factor C), and any combination thereof.

[0179] Examples of proteins associated with prion diseases may include SOD1 (superoxide dismutase 1), ALS2 (amyotrophic lateral sclerosis 2), FUS (fused in sarcoma), TARDBP (TAR DNA binding protein), VAGFA (vascular endothelial growth factor A), VAGFB (vascular endothelial growth factor B), and VAGFC (vascular endothelial growth factor C), and any combination thereof.

[0180] Examples of proteins related to neurodegenerative conditions in prion disorders may include A2M (Alpha-2-Macroglobulin), AATF (Apoptosis antagonizing transcription factor), ACPP (Acid phosphatase prostate), ACTA2 (Actin alpha 2 smooth muscle aorta), ADAM22 (ADAM metallopeptidase domain), ADORA3 (Adenosine A3 receptor), or ADRA 1D (Alpha-1 D adrenergic receptor for Alpha-1D adrenoreceptor), for example.

[0181] Examples of proteins associated with Immunodeficiency may include A2M [alpha-2-macroglobulin]; AANAT [arylalkylamine N-acetyltransferase]; ABCA1 [ATP-binding cassette, sub-family A (ABC1), member 1]; ABCA2 [ATP-binding cassette, sub-family A (ABC1), member 2]; or ABCA3 [ATP-binding cassette, sub-family A (ABC 1), member 3]; for example.

[0182] Examples of proteins associated with Trinucleotide Repeat Disorders include AR (androgen receptor), FMR1 (fragile X mental retardation 1), HTT (huntingtin), or DMPK (dystrophia myotonica-protein kinase), FXN (frataxin), ATXN2 (ataxin 2), for example.

[0183] Examples of proteins associated with Neurotransmission Disorders include SST (somatostatin), NOS 1 (nitric oxide synthase 1 (neuronal)), ADRA2A (adrenergic, alpha-2A-, receptor), ADRA2C (adrenergic, alpha-2C—, receptor), TACR1 (tachykinin receptor 1), or HTR2c (5-hydroxytryptamine (serotonin) receptor 2C), for example.

[0184] Examples of neurodevelopmental-associated sequences include A2BP1 [ataxin 2-binding protein 1], AADAT [aminoadipate aminotransferase], AANAT [arylalkylamine N-acetyltransferase], ABAT [4-aminobutyrate aminotransferase], ABCA1 [ATP-binding cassette, sub-family A (ABC1), member 1], or ABCA13 [ATP-binding cassette, sub-family A (ABC1), member 13], for example.

[0185] Further examples of preferred conditions treatable with the present system include may be selected from: Aicardi-Goutiéres Syndrome; Alexander Disease; Allan-Herndon-Dudley Syndrome; POLG-Related Disorders; Alpha-Mannosidosis (Type II and III); Alström Syndrome; Angelman; Syndrome; Ataxia-Telangiectasia; Neuronal

Ceroid-Lipofuscinoses; Beta-Thalassemia; Bilateral Optic Atrophy and (Infantile) Optic Atrophy Type 1; Retinoblastoma (bilateral); Canavan Disease; Cerebrooculofacioskeletal Syndrome 1 [COFS1]; Cerebrotendinous Xanthomatosis; Cornelia de Lange Syndrome; MAPT-Related Disorders; Genetic Prion Diseases; Dravet Syndrome; Early-Onset Familial Alzheimer Disease; Friedreich Ataxia [FRDA]; Fryns Syndrome; Fucosidosis; Fukuyama Congenital Muscular Dystrophy; Galactosialidosis; Gaucher Disease; Organic Acidemias; Hemophagocytic Lymphohistiocytosis; Hutchinson-Gilford Progeria Syndrome; Mucolipidosis II; Infantile Free Sialic Acid Storage Disease; PLA2G6-Associated Neurodegeneration; Jervell and Lange-Nielsen Syndrome; Junctional Epidermolysis Bullosa; Huntington Disease; Krabbe Disease (Infantile); Mitochondrial DNA-Associated Leigh Syndrome and NARP; Lesch-Nyhan Syndrome; LIS1-Associated Lissencephaly; Lowe Syndrome; Maple Syrup Urine Disease; MECP2 Duplication Syndrome; ATP7A-Related Copper Transport Disorders; LAMA2-Related Muscular Dystrophy; Arylsulfatase A Deficiency; Mucopolysaccharidosis Types I, II or III; Peroxisome Biogenesis Disorders, Zellweger Syndrome Spectrum; Neurodegeneration with Brain Iron Accumulation Disorders; Acid Sphingomyelinase Deficiency; Niemann-Pick Disease Type C; Glycine Encephalopathy; ARX-Related Disorders; Urea Cycle Disorders; COL1A1/2-Related Osteogenesis Imperfecta; Mitochondrial DNA Deletion Syndromes; PLP1-Related Disorders; Perry Syndrome; Phelan-McDermid Syndrome; Glycogen Storage Disease Type II (Pompe Disease) (Infantile); MAPT-Related Disorders; MECP2-Related Disorders; Rhizomelic Chondrodysplasia Punctata Type 1; Roberts Syndrome; Sandhoff Disease; Schindler Disease—Type 1; Adenosine Deaminase Deficiency; Smith-Lemli-Opitz Syndrome; Spinal Muscular Atrophy; Infantile-Onset Spinocerebellar Ataxia; Hexosaminidase A Deficiency; Thanatophoric Dysplasia Type 1; Collagen Type VI-Related Disorders; Usher Syndrome Type I; Congenital Muscular Dystrophy; Wolf-Hirschhorn Syndrome; Lysosomal Acid Lipase Deficiency; and Xeroderma Pigmentosum.

[0186] As will be apparent, it is envisaged that the present system can be used to target any polynucleotide sequence of interest. Some examples of conditions or diseases that might be usefully treated using the present system are included in the Tables above and examples of genes currently associated with those conditions are also provided there. However, the genes exemplified are not exhaustive.

EXAMPLES

[0187] The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. The present examples, along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

Example 1

CRISPR Complex Activity in the Nucleus of a Eukaryotic Cell

[0188] An example type II CRISPR system is the type II CRISPR locus from *Streptococcus pyogenes* SF370, which contains a cluster of four genes Cas9, Cas1, Cas2, and Csn1, as well as two non-coding RNA elements, tracrRNA and a characteristic array of repetitive sequences (direct repeats) interspaced by short stretches of non-repetitive sequences (spacers, about 30 bp each). In this system, targeted DNA double-strand break (DSB) is generated in four sequential steps (FIG. **2**A). First, two non-coding RNAs, the pre-crRNA array and tracrRNA, are transcribed from the CRISPR locus. Second, tracrRNA hybridizes to the direct repeats of pre-crRNA, which is then processed into mature crRNAs containing individual spacer sequences. Third, the mature crRNA: tracrRNA complex directs Cas9 to the DNA target consisting of the protospacer and the corresponding PAM via heteroduplex formation between the spacer region of the crRNA and the protospacer DNA. Finally, Cas9 mediates cleavage of target DNA upstream of PAM to create a DSB within the protospacer (FIG. **2**A). This example describes an example process for adapting this RNA-programmable nuclease system to direct CRISPR complex activity in the nuclei of eukaryotic cells.

[0189] Cell Culture and Transfection

[0190] Human embryonic kidney (HEK) cell line HEK 293FT (Life Technologies) was maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone), 2 mM GlutaMAX (Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° C. with 5% CO_2 incubation. Mouse neuro2A (N2A) cell line (ATCC) was maintained with DMEM supplemented with 5% fetal bovine serum (HyClone), 2 mM GlutaMAX (Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° C. with 5% CO_2.

[0191] HEK 293FT or N2A cells were seeded into 24-well plates (Corning) one day prior to transfection at a density of 200,000 cells per well. Cells were transfected using Lipofectamine 2000 (Life Technologies) following the manufacturer's recommended protocol. For each well of a 24-well plate a total of 800 ng of plasmids were used.

[0192] Surveyor Assay and Sequencing Analysis for Genome Modification

[0193] HEK 293FT or N2A cells were transfected with plasmid DNA as described above. After transfection, the cells were incubated at 37° C. for 72 hours before genomic DNA extraction. Genomic DNA was extracted using the QuickExtract DNA extraction kit (Epicentre) following the manufacturer's protocol. Briefly, cells were resuspended in QuickExtract solution and incubated at 65° C. for 15 minutes and 98° C. for 10 minutes. Extracted genomic DNA was immediately processed or stored at −20° C.

[0194] The genomic region surrounding a CRISPR target site for each gene was PCR amplified, and products were purified using QiaQuick Spin Column (Qiagen) following manufacturer's protocol. A total of 400 ng of the purified PCR products were mixed with 2 μl 10× Tag polymerase PCR buffer (Enzymatics) and ultrapure water to a final volume of 20 μl, and subjected to a re-annealing process to enable heteroduplex formation: 95° C. for 10 min, 95° C. to 85° C. ramping at −2° C./s, 85° C. to 25° C. at −0.25° C./s, and 25° C. hold for 1 minute. After re-annealing, products were treated with Surveyor nuclease and Surveyor enhancer S (Transgenomics) following the manufacturer's recommended protocol, and analyzed on 4-20% Novex TBE polyacrylamide gels (Life Technologies). Gels were stained with SYBR Gold DNA stain (Life Technologies) for 30 minutes and imaged with a Gel Doc gel imaging system (Bio-rad).

Quantification was based on relative band intensities, as a measure of the fraction of cleaved DNA. FIG. **8** provides a schematic illustration of this Surveyor assay.

[0195] Restriction Fragment Length Polymorphism Assay for Detection of Homologous Recombination

[0196] HEK 293FT and N2A cells were transfected with plasmid DNA, and incubated at 37° C. for 72 hours before genomic DNA extraction as described above. The target genomic region was PCR amplified using primers outside the homology arms of the homologous recombination (HR) template. PCR products were separated on a 1% agarose gel and extracted with MinElute GelExtraction Kit (Qiagen). Purified products were digested with HindIII (Fermentas) and analyzed on a 6% Novex TBE poly-acrylamide gel (Life Technologies).

[0197] RNA Secondary Structure Prediction and Analysis

[0198] RNA secondary structure prediction was performed using the online webserver RNAfold developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g. A. R. Gruber et al., 2008, Cell 106(1): 23-24; and PA Can and GM Church, 2009, Nature Biotechnology 27(12): 1151-62).

[0199] Bacterial Plasmid Transformation Interference Assay

[0200] Elements of the *S. pyogenes* CRISPR locus 1 sufficient for CRISPR activity were reconstituted in *E. coli* using pCRISPR plasmid (schematically illustrated in FIG. **10**A). pCRISPR contained tracrRNA, SpCas9, and a leader sequence driving the crRNA array. Spacers (also referred to as "guide sequences") were inserted into the crRNA array between BsaI sites using annealed oligonucleotides, as illustrated. Challenge plasmids used in the interference assay were constructed by inserting the protospacer (also referred to as a "target sequence") sequence along with an adjacent CRISPR motif sequence (PAM) into pUC19 (see FIG. **10**B). The challenge plasmid contained ampicillin resistance. FIG. **10**C provides a schematic representation of the interference assay. Chemically competent *E. coli* strains already carrying pCRISPR and the appropriate spacer were transformed with the challenge plasmid containing the corresponding protospacer-PAM sequence. pUC19 was used to assess the transformation efficiency of each pCRISPR-carrying competent strain. CRISPR activity resulted in cleavage of the pPSP plasmid carrying the protospacer, precluding ampicillin resistance otherwise conferred by pUC 19 lacking the protospacer. FIG. **10**D illustrates competence of each pCRISPR-carrying *E. coli* strain used in assays illustrated in FIG. **4**C.

[0201] RNA Purification

[0202] HEK 293FT cells were maintained and transfected as stated above. Cells were harvested by trypsinization followed by washing in phosphate buffered saline (PBS). Total cell RNA was extracted with TRI reagent (Sigma) following manufacturer's protocol. Extracted total RNA was quantified using Naonodrop (Thermo Scientific) and normalized to same concentration.

[0203] Northern Blot Analysis of crRNA and tracrRNA Expression in Mammalian Cells

[0204] RNAs were mixed with equal volumes of 2× loading buffer (Ambion), heated to 95° C. for 5 min, chilled on ice for 1 min, and then loaded onto 8% denaturing polyacrylamide gels (SequaGel, National Diagnostics) after pre-running the gel for at least 30 minutes. The samples were electrophoresed for 1.5 hours at 40 W limit. Afterwards, the RNA was transferred to Hybond N+membrane (GE Healthcare) at 300 mA in a semi-dry transfer apparatus (Bio-rad) at room temperature for 1.5 hours. The RNA was crosslinked to the membrane using autocrosslink button on Stratagene UV Crosslinker the Stratalinker (Stratagene). The membrane was pre-hybridized in ULTRAhyb-Oligo Hybridization Buffer (Ambion) for 30 min with rotation at 42° C., and probes were then added and hybridized overnight. Probes were ordered from IDT and labeled with [gamma-^{32}P] ATP (Perkin Elmer) with T4 polynucleotide kinase (New England Biolabs). The membrane was washed once with pre-warmed (42° C.) 2×SSC, 0.5% SDS for 1 min followed by two 30 minute washes at 42° C. The membrane was exposed to a phosphor screen for one hour or overnight at room temperature and then scanned with a phosphorimager (Typhoon).

[0205] Bacterial CRISPR System Construction and Evaluation

[0206] CRISPR locus elements, including tracrRNA, Cas9, and leader were PCR amplified from *Streptococcus pyogenes* SF370 genomic DNA with flanking homology arms for Gibson Assembly. Two BsaI type IIS sites were introduced in between two direct repeats to facilitate easy insertion of spacers (FIG. **9**). PCR products were cloned into EcoRV-digested pACYC 184 downstream of the tet promoter using Gibson Assembly Master Mix (NEB). Other endogenous CRISPR system elements were omitted, with the exception of the last 50 bp of Csn2. Oligos (Integrated DNA Technology) encoding spacers with complimentary overhangs were cloned into the BsaI-digested vector pDC000 (NEB) and then ligated with T7 ligase (Enzymatics) to generate pCRISPR plasmids. Challenge plasmids containing spacers with PAM sequences (also referred to herein as "CRISPR motif sequences") were created by ligating hybridized oligos carrying compatible overhangs (Integrated DNA Technology) into BamHI-digested pUC19. Cloning for all constructs was performed in *E. coli* strain JM109 (Zymo Research).

[0207] pCRISPR-carrying cells were made competent using the Z-Competent *E. coli* Transformation Kit and Buffer Set (Zymo Research, T3001) according to manufacturer's instructions. In the transformation assay, 50 uL aliquots of competent cells carrying pCRISPR were thawed on ice and transformed with 1 ng of spacer plasmid or pUC19 on ice for 30 minutes, followed by 45 second heat shock at 42° C. and 2 minutes on ice. Subsequently, 250 ul SOC (Invitrogen) was added followed by shaking incubation at 37° C. for 1 hr, and 100 uL of the post-SOC outgrowth was plated onto double selection plates (12.5 ug/ml chloramphenicol, 100 ug/ml ampicillin). To obtain cfu/ng of DNA, total colony numbers were multiplied by 3.

[0208] To improve expression of CRISPR components in mammalian cells, two genes from the SF370 locus 1 of *Streptococcus pyogenes* (*S. pyogenes*) were codon-optimized, Cas9 (SpCas9) and RNase III (SpRNase III). To facilitate nuclear localization, a nuclear localization signal (NLS) was included at the amino (N)- or carboxyl (C)-termini of both SpCas9 and SpRNase III (FIG. **2**B). To facilitate visualization of protein expression, a fluorescent protein marker was also included at the N- or C-termini of both proteins (FIG. **2**B). A version of SpCas9 with an NLS attached to both N- and C-termini (2xNLS-SpCas9) was also generated. Constructs containing NLS-fused SpCas9 and SpRNase III were transfected into 293FT human embryonic kidney (HEK) cells, and the relative positioning of the NLS to SpCas9 and SpRNase III was found to affect their nuclear localization efficiency. Whereas the C-terminal NLS was sufficient to target SpR-

Nase III to the nucleus, attachment of a single copy of these particular NLS's to either the N- or C-terminus of SpCas9 was unable to achieve adequate nuclear localization in this system. In this example, the C-terminal NLS was that of nucleoplasmin (KRPAATKKAGQAKKKK (SEQ ID NO: 2)), and the C-terminal NLS was that of the SV40 large T-antigen (PKKKRKV (SEQ ID NO: 1)). Of the versions of SpCas9 tested, only 2xNLS-SpCas9 exhibited nuclear localization (FIG. **2**B).

[0209] The tracrRNA from the CRISPR locus of *S. pyogenes* SF370 has two transcriptional start sites, giving rise to two transcripts of 89-nucleotides (nt) and 171 nt that are subsequently processed into identical 75 nt mature tracrRNAs. The shorter 89 nt tracrRNA was selected for expression in mammalian cells (expression constructs illustrated in FIG. **7**A, with functionality as determined by results of the Surveyor assay shown in FIG. **7**B). Transcription start sites are marked as +1, and transcription terminator and the sequence probed by northern blot are also indicated. Expression of processed tracrRNA was also confirmed by Northern blot. FIG. **7**C shows results of a Northern blot analysis of total RNA extracted from 293FT cells transfected with U6 expression constructs carrying long or short tracrRNA, as well as SpCas9 and DR-EMX1(1)-DR. Left and right panels are from 293FT cells transfected without or with SpRNase III, respectively. U6 indicate loading control blotted with a probe targeting human U6 snRNA. Transfection of the short tracrRNA expression construct led to abundant levels of the processed form of tracrRNA (~75 bp). Very low amounts of long tracrRNA are detected on the Northern blot.

[0210] To promote precise transcriptional initiation, the RNA polymerase III-based U6 promoter was selected to drive the expression of tracrRNA (FIG. **2**C). Similarly, a U6 promoter-based construct was developed to express a pre-crRNA array consisting of a single spacer flanked by two direct repeats (DRs, also encompassed by the term "tracr-mate sequences"; FIG. **2**C). The initial spacer was designed to target a 33-base-pair (bp) target site (30-bp protospacer plus a 3-bp CRISPR motif (PAM) sequence satisfying the NGG recognition motif of Cas9) in the human EMX1 locus (FIG. **2**C), a key gene in the development of the cerebral cortex.

[0211] To test whether heterologous expression of the CRISPR system (SpCas9, SpRNase III, tracrRNA, and pre-crRNA) in mammalian cells can achieve targeted cleavage of mammalian chromosomes, HEK 293FT cells were transfected with combinations of CRISPR components. Since DSBs in mammalian nuclei are partially repaired by the non-homologous end joining (NHEJ) pathway, which leads to the formation of indels, the Surveyor assay was used to detect potential cleavage activity at the target EMX1 locus (FIG. **8**) (see e.g. Guschin et al., 2010, Methods Mol Biol 649: 247). Co-transfection of all four CRISPR components was able to induce up to 5.0% cleavage in the protospacer (see FIG. **2**D). Co-transfection of all CRISPR components minus SpRNase III also induced up to 4.7% indel in the protospacer, suggesting that there may be endogenous mammalian RNases that are capable of assisting with crRNA maturation, such as for example the related Dicer and Drosha enzymes. Removing any of the remaining three components abolished the genome cleavage activity of the CRISPR system (FIG. **2**D). Sanger sequencing of amplicons containing the target locus verified the cleavage activity: in 43 sequenced clones, 5 mutated alleles (11.6%) were found. Similar experiments using a variety of guide sequences produced indel percentages as high as 29% (see FIGS. **4**-**7**, **12**, and **13**). These results define a three-component system for efficient CRISPR-mediated genome modification in mammalian cells. To optimize the cleavage efficiency, Applicants also tested whether different isoforms of tracrRNA affected the cleavage efficiency and found that, in this example system, only the short (89-bp) transcript form was able to mediate cleavage of the human EMX1 genomic locus (FIG. **7**B).

[0212] FIG. **14** provides an additional Northern blot analysis of crRNA processing in mammalian cells. FIG. **14**A illustrates a schematic showing the expression vector for a single spacer flanked by two direct repeats (DR-EMX1(1)-DR). The 30 bp spacer targeting the human EMX1 locus protospacer 1 (see FIG. **6**) and the direct repeat sequences are shown in the sequence beneath FIG. **14**A. The line indicates the region whose reverse-complement sequence was used to generate Northern blot probes for EMX1(1) crRNA detection. FIG. **14**B shows a Northern blot analysis of total RNA extracted from 293FT cells transfected with U6 expression constructs carrying DR-EMX1(1)-DR. Left and right panels are from 293FT cells transfected without or with SpRNase III respectively. DR-EMX 1(1)-DR was processed into mature crRNAs only in the presence of SpCas9 and short tracrRNA and was not dependent on the presence of SpRNase III. The mature crRNA detected from transfected 293 FT total RNA is ~33 bp and is shorter than the 39-42 bp mature crRNA from *S. pyogenes*. These results demonstrate that a CRISPR system can be transplanted into eukaryotic cells and reprogrammed to facilitate cleavage of endogenous mammalian target polynucleotides.

[0213] FIG. **2** illustrates the bacterial CRISPR system described in this example. FIG. **2**A illustrates a schematic showing the CRISPR locus 1 from *Streptococcus pyogenes* SF370 and a proposed mechanism of CRISPR-mediated DNA cleavage by this system. Mature crRNA processed from the direct repeat-spacer array directs Cas9 to genomic targets consisting of complimentary protospacers and a protospacer-adjacent motif (PAM). Upon target-spacer base pairing, Cas9 mediates a double-strand break in the target DNA. FIG. **2**B illustrates engineering of *S. pyogenes* Cas9 (SpCas9) and RNase III (SpRNase III) with nuclear localization signals (NLSs) to enable import into the mammalian nucleus. FIG. **2**C illustrates mammalian expression of SpCas9 and SpRNase III driven by the constitutive EF1a promoter and tracrRNA and pre-crRNA array (DR-Spacer-DR) driven by the RNA Pol3 promoter U6 to promote precise transcription initiation and termination. A protospacer from the human EMX1 locus with a satisfactory PAM sequence is used as the spacer in the pre-crRNA array. FIG. **2**D illustrates surveyor nuclease assay for SpCas9-mediated minor insertions and deletions. SpCas9 was expressed with and without SpRNase III, tracrRNA, and a pre-crRNA array carrying the EMX1-target spacer. FIG. **2**E illustrates a schematic representation of base pairing between target locus and EMX1-targeting crRNA, as well as an example chromatogram showing a micro deletion adjacent to the SpCas9 cleavage site. FIG. **2**F illustrates mutated alleles identified from sequencing analysis of 43 clonal amplicons showing a variety of micro insertions and deletions. Dashes indicate deleted bases, and non-aligned or mismatched bases indicate insertions or mutations. Scale bar=10 μm.

[0214] To further simplify the three-component system, a chimeric crRNA-tracrRNA hybrid design was adapted, where a mature crRNA (comprising a guide sequence) is

fused to a partial tracrRNA via a stem-loop to mimic the natural crRNA:tracrRNA duplex (FIG. **3**A). To increase co-delivery efficiency, a bicistronic expression vector was created to drive co-expression of a chimeric RNA and SpCas9 in transfected cells (FIGS. **3**A and **8**). In parallel, the bicistronic vectors were used to express a pre-crRNA (DR-guide sequence-DR) with SpCas9, to induce processing into crRNA with a separately expressed tracrRNA (compare FIG. **13**B top and bottom). FIG. **9** provides schematic illustrations of bicistronic expression vectors for pre-crRNA array (FIG. **9**A) or chimeric crRNA (represented by the short line downstream of the guide sequence insertion site and upstream of the EF1α promoter in FIG. **9**B) with hSpCas9, showing location of various elements and the point of guide sequence insertion. The expanded sequence around the location of the guide sequence insertion site in FIG. **9**B also shows a partial DR sequence (GTTTTAGAGCTA (SEQ ID NO: 27)) and a partial tracrRNA sequence (TAGCAAGTTAAAATAAGGCTAGTCCGTTTTT (SEQ ID NO: 28)). Guide sequences can be inserted between BbsI sites using annealed oligonucleotides. Sequence design for the oligonucleotides are shown below the schematic illustrations in FIG. **9**, with appropriate ligation adapters indicated. WPRE represents the Woodchuck hepatitis virus post-transcriptional regulatory element. The efficiency of chimeric RNA-mediated cleavage was tested by targeting the same EMX1 locus described above. Using both Surveyor assay and Sanger sequencing of amplicons, Applicants confirmed that the chimeric RNA design facilitates cleavage of human EMX1 locus with approximately a 4.7% modification rate (FIG. **4**).

[0215] Generalizability of CRISPR-mediated cleavage in eukaryotic cells was tested by targeting additional genomic loci in both human and mouse cells by designing chimeric RNA targeting multiple sites in the human EMX1 and PVALB, as well as the mouse Th loci. FIG. **15** illustrates the selection of some additional targeted protospacers in human PVALB (FIG. **15**A) and mouse Th (FIG. **15**B) loci. Schematics of the gene loci and the location of three protospacers within the last exon of each are provided. The underlined sequences include 30 bp of protospacer sequence and 3 bp at the 3' end corresponding to the PAM sequences. Protospacers on the sense and anti-sense strands are indicated above and below the DNA sequences, respectively. A modification rate of 6.3% and 0.75% was achieved for the human PVALB and mouse Th loci respectively, demonstrating the broad applicability of the CRISPR system in modifying different loci across multiple organisms (FIGS. **3**B and **6**). While cleavage was only detected with one out of three spacers for each locus using the chimeric constructs, all target sequences were cleaved with efficiency of indel production reaching 27% when using the co-expressed pre-crRNA arrangement (FIG. **6**).

[0216] FIG. **13** provides a further illustration that SpCas9 can be reprogrammed to target multiple genomic loci in mammalian cells. FIG. **13**A provides a schematic of the human EMX1 locus showing the location of five protospacers, indicated by the underlined sequences. FIG. **13**B provides a schematic of the pre-crRNA/trcrRNA complex showing hybridization between the direct repeat region of the pre-crRNA and tracrRNA (top), and a schematic of a chimeric RNA design comprising a 20 bp guide sequence, and tracr mate and tracr sequences consisting of partial direct repeat and tracrRNA sequences hybridized in a hairpin structure (bottom). Results of a Surveyor assay comparing the efficacy of Cas9-mediated cleavage at five protospacers in the human EMX1 locus is illustrated in FIG. **13**C. Each protospacer is targeted using either processed pre-crRNA/tracrRNA complex (crRNA) or chimeric RNA (chiRNA).

[0217] Since the secondary structure of RNA can be crucial for intermolecular interactions, a structure prediction algorithm based on minimum free energy and Boltzmann-weighted structure ensemble was used to compare the putative secondary structure of all guide sequences used in our genome targeting experiment (FIG. **3**B) (see e.g. Gruber et al., 2008, Nucleic Acids Research, 36: W70). Analysis revealed that in most cases, the effective guide sequences in the chimeric crRNA context were substantially free of secondary structure motifs, whereas the ineffective guide sequences were more likely to form internal secondary structures that could prevent base pairing with the target protospacer DNA. It is thus possible that variability in the spacer secondary structure might impact the efficiency of CRISPR-mediated interference when using a chimeric crRNA.

[0218] FIG. **3** illustrates example expression vectors. FIG. **3**A provides a schematic of a bi-cistronic vector for driving the expression of a synthetic crRNA-tracrRNA chimera (chimeric RNA) as well as SpCas9. The chimeric guide RNA contains a 20-bp guide sequence corresponding to the protospacer in the genomic target site. FIG. **3**B provides a schematic showing guide sequences targeting the human EMX1, PVALB, and mouse Th loci, as well as their predicted secondary structures. The modification efficiency at each target site is indicated below the RNA secondary structure drawing (EMX1, n=216 amplicon sequencing reads; PVALB, n=224 reads; Th, n=265 reads). The folding algorithm produced an output with each base colored according to its probability of assuming the predicted secondary structure, as indicated by a rainbow scale that is reproduced in FIG. **3**B in gray scale. Further vector designs for SpCas9 are shown in FIG. **44**, which illustrates single expression vectors incorporating a U6 promoter linked to an insertion site for a guide oligo, and a Cbh promoter linked to SpCas9 coding sequence. The vector shown in FIG. **44***b* includes a tracrRNA coding sequence linked to an H1 promoter.

[0219] To test whether spacers containing secondary structures are able to function in prokaryotic cells where CRISPRs naturally operate, transformation interference of protospacer-bearing plasmids were tested in an *E. coli* strain heterologously expressing the *S. pyogenes* SF370 CRISPR locus 1 (FIG. **10**). The CRISPR locus was cloned into a low-copy *E. coli* expression vector and the crRNA array was replaced with a single spacer flanked by a pair of DRs (pCRISPR). *E. coli* strains harboring different pCRISPR plasmids were transformed with challenge plasmids containing the corresponding protospacer and PAM sequences (FIG. **10**C). In the bacterial assay, all spacers facilitated efficient CRISPR interference (FIG. **4**C). These results suggest that there may be additional factors affecting the efficiency of CRISPR activity in mammalian cells.

[0220] To investigate the specificity of CRISPR-mediated cleavage, the effect of single-nucleotide mutations in the guide sequence on protospacer cleavage in the mammalian genome was analyzed using a series of EMX1-targeting chimeric crRNAs with single point mutations (FIG. **4**A). FIG. **4**B illustrates results of a Surveyor nuclease assay comparing the cleavage efficiency of Cas9 when paired with different mutant chimeric RNAs. Single-base mismatch up to 12-bp 5'

of the PAM substantially abrogated genomic cleavage by SpCas9, whereas spacers with mutations at farther upstream positions retained activity against the original protospacer target (FIG. **4**B). In addition to the PAM, SpCas9 has single-base specificity within the last 12-bp of the spacer. Furthermore, CRISPR is able to mediate genomic cleavage as efficiently as a pair of TALE nucleases (TALEN) targeting the same EMX1 protospacer. FIG. **4**C provides a schematic showing the design of TALENs targeting EMX1, and FIG. **4**D shows a Surveyor gel comparing the efficiency of TALEN and Cas9 (n=3).

[0221] Having established a set of components for achieving CRISPR-mediated gene editing in mammalian cells through the error-prone NHEJ mechanism, the ability of CRISPR to stimulate homologous recombination (HR), a high fidelity gene repair pathway for making precise edits in the genome, was tested. The wild type SpCas9 is able to mediate site-specific DSBs, which can be repaired through both NHEJ and HR. In addition, an aspartate-to-alanine substitution (D 10A) in the RuvC I catalytic domain of SpCas9 was engineered to convert the nuclease into a nickase (SpCas9n; illustrated in FIG. **5**A) (see e.g. Sapranauskas et al., 2011, Nucleic Acids Research, 39: 9275; Gasiunas et al., 2012, Proc. Natl. Acad. Sci. USA, 109:E2579), such that nicked genomic DNA undergoes the high-fidelity homology-directed repair (HDR). Surveyor assay confirmed that SpCas9n does not generate indels at the EMX1 protospacer target. As illustrated in FIG. **5**B, co-expression of EMX1-targeting chimeric crRNA with SpCas9 produced indels in the target site, whereas co-expression with SpCas9n did not (n=3). Moreover, sequencing of 327 amplicons did not detect any indels induced by SpCas9n. The same locus was selected to test CRISPR-mediated HR by co-transfecting HEK 293FT cells with the chimeric RNA targeting EMX1, hSpCas9 or hSpCas9n, as well as a HR template to introduce a pair of restriction sites (HindIII and NheI) near the protospacer. FIG. **5**C provides a schematic illustration of the HR strategy, with relative locations of recombination points and primer annealing sequences (arrows). SpCas9 and SpCas9n indeed catalyzed integration of the HR template into the EMX1 locus. PCR amplification of the target region followed by restriction digest with HindIII revealed cleavage products corresponding to expected fragment sizes (arrows in restriction fragment length polymorphism gel analysis shown in FIG. **5**D), with SpCas9 and SpCas9n mediating similar levels of HR efficiencies. Applicants further verified HR using Sanger sequencing of genomic amplicons (FIG. **5**E). These results demonstrate the utility of CRISPR for facilitating targeted gene insertion in the mammalian genome. Given the 14-bp (12-bp from the spacer and 2-bp from the PAM) target specificity of the wild type SpCas9, the availability of a nickase can significantly reduce the likelihood of off-target modifications, since single strand breaks are not substrates for the error-prone NHEJ pathway.

[0222] Expression constructs mimicking the natural architecture of CRISPR loci with arrayed spacers (FIG. **2**A) were constructed to test the possibility of multiplexed sequence targeting. Using a single CRISPR array encoding a pair of EMX1- and PVALB-targeting spacers, efficient cleavage at both loci was detected (FIG. **4**F, showing both a schematic design of the crRNA array and a Surveyor blot showing efficient mediation of cleavage). Targeted deletion of larger genomic regions through concurrent DSBs using spacers against two targets within EMX1 spaced by 119 bp was also tested, and a 1.6% deletion efficacy (3 out of 182 amplicons; FIG. **4**G) was detected. This demonstrates that the CRISPR system can mediate multiplexed editing within a single genome.

Example 2

CRISPR System Modifications and Alternatives

[0223] The ability to use RNA to program sequence-specific DNA cleavage defines a new class of genome engineering tools for a variety of research and industrial applications. Several aspects of the CRISPR system can be further improved to increase the efficiency and versatility of CRISPR targeting. Optimal Cas9 activity may depend on the availability of free Mg^{2+} at levels higher than that present in the mammalian nucleus (see e.g. Jinek et al., 2012, Science, 337:816), and the preference for an NGG motif immediately downstream of the protospacer restricts the ability to target on average every 12-bp in the human genome (FIG. **11**, evaluating both plus and minus strands of human chromosomal sequences). Some of these constraints can be overcome by exploring the diversity of CRISPR loci across the microbial metagenome (see e.g. Makarova et al., 2011, Nat Rev Microbiol, 9:467). Other CRISPR loci may be transplanted into the mammalian cellular milieu by a process similar to that described in Example 1. For example, FIG. **12** illustrates adaptation of the Type II CRISPR system from CRISPR 1 of *Streptococcus thermophilus* LMD-9 for heterologous expression in mammalian cells to achieve CRISPR-mediated genome editing. FIG. **12**A provides a Schematic illustration of CRISPR 1 from *S. thermophilus* LMD-9. FIG. **12**B illustrates the design of an expression system for the *S. thermophilus* CRISPR system. Human codon-optimized hStCas9 is expressed using a constitutive EF1α promoter. Mature versions of tracrRNA and crRNA are expressed using the U6 promoter to promote precise transcription initiation. Sequences from the mature crRNA and tracrRNA are illustrated. A single base indicated by the lower case "a" in the crRNA sequence is used to remove the polyU sequence, which serves as a RNA polIII transcriptional terminator. FIG. **12**C provides a schematic showing guide sequences targeting the human EMX1 locus as well as their predicted secondary structures. The modification efficiency at each target site is indicated below the RNA secondary structures. The algorithm generating the structures colors each base according to its probability of assuming the predicted secondary structure, which is indicated by a rainbow scale reproduced in FIG. **12**C in gray scale. FIG. **12**D shows the results of hStCas9-mediated cleavage in the target locus using the Surveyor assay. RNA guide spacers 1 and 2 induced 14% and 6.4%, respectively. Statistical analysis of cleavage activity across biological replica at these two protospacer sites is also provided in FIG. **6**. FIG. **16** provides a schematic of additional protospacer and corresponding PAM sequence targets of the *S. thermophilus* CRISPR system in the human EMX1 locus. Two protospacer sequences are highlighted and their corresponding PAM sequences satisfying NNAGAAW motif are indicated by underlining 3' with respect to the corresponding highlighted sequence. Both protospacers target the anti-sense strand.

Example 3

Sample Target Sequence Selection Algorithm

[0224] A software program is designed to identify candidate CRISPR target sequences on both strands of an input

DNA sequence based on desired guide sequence length and a CRISPR motif sequence (PAM) for a specified CRISPR enzyme. For example, target sites for Cas9 from *S. pyogenes*, with PAM sequences NGG, may be identified by searching for 5'-N_x-NGG-3' both on the input sequence and on the reverse-complement of the input. Likewise, target sites for Cas9 of *S. thermophilus* CRISPR1, with PAM sequence NNAGAAW, may be identified by searching for 5'-N_x-NNAGAAW-3' (SEQ ID NO: 29) both on the input sequence and on the reverse-complement of the input. Likewise, target sites for Cas9 of *S. thermophilus* CRISPR3, with PAM sequence NGGNG, may be identified by searching for 5'-N_x-NGGNG-3' both on the input sequence and on the reverse-complement of the input. The value "x" in N_x may be fixed by the program or specified by the user, such as 20.

[0225] Since multiple occurrences in the genome of the DNA target site may lead to nonspecific genome editing, after identifying all potential sites, the program filters out sequences based on the number of times they appear in the relevant reference genome. For those CRISPR enzymes for which sequence specificity is determined by a 'seed' sequence, such as the 11-12 bp 5' from the PAM sequence, including the PAM sequence itself, the filtering step may be based on the seed sequence. Thus, to avoid editing at additional genomic loci, results are filtered based on the number of occurrences of the seed:PAM sequence in the relevant genome. The user may be allowed to choose the length of the seed sequence. The user may also be allowed to specify the number of occurrences of the seed:PAM sequence in a genome for purposes of passing the filter. The default is to screen for unique sequences. Filtration level is altered by changing both the length of the seed sequence and the number of occurrences of the sequence in the genome. The program may in addition or alternatively provide the sequence of a guide sequence complementary to the reported target sequence(s) by providing the reverse complement of the identified target sequence(s).

[0226] Further details of methods and algorithms to optimize sequence selection can be found in U.S. application Ser. No. 61/836,080 (attorney docket 44790.11.2022); incorporated herein by reference.

Example 4

Evaluation of Multiple Chimeric crRNA-tracrRNA Hybrids

[0227] This example describes results obtained for chimeric RNAs (chiRNAs; comprising a guide sequence, a tracr mate sequence, and a tracr sequence in a single transcript) having tracr sequences that incorporate different lengths of wild-type tracrRNA sequence. FIG. **18***a* illustrates a schematic of a bicistronic expression vector for chimeric RNA and Cas9. Cas9 is driven by the CBh promoter and the chimeric RNA is driven by a U6 promoter. The chimeric guide RNA consists of a 20 bp guide sequence (Ns) joined to the tracr sequence (running from the first "U" of the lower strand to the end of the transcript), which is truncated at various positions as indicated. The guide and tracr sequences are separated by the tracr-mate sequence GUUUUAGAGCUA (SEQ ID NO: 30) followed by the loop sequence GAAA. Results of SURVEYOR assays for Cas9-mediated indels at the human EMX1 and PVALB loci are illustrated in FIGS. **18***b* and **18***c*, respectively. Arrows indicate the expected SURVEYOR fragments. ChiRNAs are indicated by their "+n" designation, and crRNA refers to a hybrid RNA where guide and tracr sequences are expressed as separate transcripts. Quantification of these results, performed in triplicate, are illustrated by histogram in FIGS. **19***a* and **19***b*, corresponding to FIGS. **18***b* and **18***c*, respectively ("N.D." indicates no indels detected). Protospacer IDs and their corresponding genomic target, protospacer sequence, PAM sequence, and strand location are provided in Table D. Guide sequences were designed to be complementary to the entire protospacer sequence in the case of separate transcripts in the hybrid system, or only to the underlined portion in the case of chimeric RNAs.

TABLE D

protospacer ID	genomic target	protospacer sequence (5' to 3')	PAM	strand
1	EMX1	GGACATCGATGTCACCTCCAATGACT (SEQ ID NO: 31)	TGG AGGG	+
2	EMX1	CATTGGAGGTGACATCGATGTCCTCC (SEQ ID NO: 32)	TGG CCAT	-
3	EMX1	GGAAGGGCCTGAGTCCGAGCAGAAGA (SEQ ID NO: 33)	GGG AGAA	+
4	PVALB	GGTGGCGAGAGGGGCCGAGATTGGGT (SEQ ID NO: 34)	AGG GTTC	+
5	PVALB	ATGCAGGAGGGTGGCGAGAGGGGCCG (SEQ ID NO: 35)	TGG AGAT	+

[0228] Cell Culture and Transfection

[0229] Human embryonic kidney (HEK) cell line 293FT (Life Technologies) was maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone), 2 mM GlutaMAX (Life Technologies), 100 U/mL penicillin, and 100 μn/mL streptomycin at 37° C. with 5% CO_2 incubation. 293FT cells were seeded onto 24-well plates (Corning) 24 hours prior to transfection at a density of 150,000 cells per well. Cells were transfected using Lipofectamine 2000 (Life Technologies) following the manufacturer's recommended protocol. For each well of a 24-well plate, a total of 500 ng plasmid was used.

[0230] SURVEYOR Assay for Genome Modification

[0231] 293FT cells were transfected with plasmid DNA as described above. Cells were incubated at 37° C. for 72 hours post-transfection prior to genomic DNA extraction. Genomic DNA was extracted using the QuickExtract DNA Extraction Solution (Epicentre) following the manufacturer's protocol. Briefly, pelleted cells were resuspended in QuickExtract solution and incubated at 65° C. for 15 minutes and 98° C. for 10 minutes. The genomic region flanking the CRISPR target site for each gene was PCR amplified (primers listed in Table E), and products were purified using QiaQuick Spin Column (Qiagen) following the manufacturer's protocol. 400 ng total of the purified PCR products were mixed with 2 μl 10× Taq DNA Polymerase PCR buffer (Enzymatics) and ultrapure water to a final volume of 20 μl, and subjected to a re-annealing process to enable heteroduplex formation: 95° C. for 10 min, 95° C. to 85° C. ramping at −2° C./s, 85° C. to 25° C. at −0.25° C./s, and 25° C. hold for 1 minute. After re-annealing, products were treated with SURVEYOR nuclease and SURVEYOR enhancer S (Transgenomics) following the manufacturer's recommended protocol, and analyzed on 4-20% Novex TBE poly-acrylamide gels (Life Technologies). Gels were stained with SYBR Gold DNA stain (Life Technolo-

gies) for 30 minutes and imaged with a Gel Doc gel imaging system (Bio-rad). Quantification was based on relative band intensities.

TABLE E

primer name	genomic target	primer sequence (5' to 3')
Sp-EMX1-F	EMX1	AAAACCACCCTTCTCTCTGGC (SEQ ID NO: 36)
Sp-EMX1-R	EMX1	GGAGATTGGAGACACGGAGA G (SEQ ID NO: 37)
Sp-PVALB-F	PVALB	CTGGAAAGCCAATGCCTGAC (SEQ ID NO: 38)
Sp-PVALB-R	PVALB	GGCAGCAAACTCCTTGTCCT (SEQ ID NO: 39)

[0232] Computational Identification of Unique CRISPR Target Sites

[0233] To identify unique target sites for the *S. pyogenes* SF370 Cas9 (SpCas9) enzyme in the human, mouse, rat, zebrafish, fruit fly, and *C. elegans* genome, we developed a software package to scan both strands of a DNA sequence and identify all possible SpCas9 target sites. For this example, each SpCas9 target site was operationally defined as a 20 bp sequence followed by an NGG protospacer adjacent motif (PAM) sequence, and we identified all sequences satisfying this 5'-N_{20}—NGG-3' definition on all chromosomes. To prevent non-specific genome editing, after identifying all potential sites, all target sites were filtered based on the number of times they appear in the relevant reference genome. To take advantage of sequence specificity of Cas9 activity conferred by a 'seed' sequence, which can be, for example, approximately 11-12 bp sequence 5' from the PAM sequence, 5'-NNNNNNNNN-NGG-3' sequences were selected to be unique in the relevant genome. All genomic sequences were downloaded from the UCSC Genome Browser (Human genome hg19, Mouse genome mm9, Rat genome rn5, Zebrafish genome danRer7, *D. melanogaster* genome dm4 and *C. elegans* genome ce10). The full search results are available to browse using UCSC Genome Browser information. An example visualization of some target sites in the human genome is provided in FIG. **21**.

[0234] Initially, three sites within the EMX1 locus in human HEK 293FT cells were targeted. Genome modification efficiency of each chiRNA was assessed using the SURVEYOR nuclease assay, which detects mutations resulting from DNA double-strand breaks (DSBs) and their subsequent repair by the non-homologous end joining (NHEJ) DNA damage repair pathway. Constructs designated chiRNA(+n) indicate that up to the +n nucleotide of wild-type tracrRNA is included in the chimeric RNA construct, with values of 48, 54, 67, and 85 used for n. Chimeric RNAs containing longer fragments of wild-type tracrRNA (chiRNA(+67) and chiRNA(+85)) mediated DNA cleavage at all three EMX1 target sites, with chiRNA(+85) in particular demonstrating significantly higher levels of DNA cleavage than the corresponding crRNA/tracrRNA hybrids that expressed guide and tracr sequences in separate transcripts (FIGS. **18***b* and **19***a*). Two sites in the PVALB locus that yielded no detectable cleavage using the hybrid system (guide sequence and tracr sequence expressed as separate transcripts) were also targeted using chiRNAs. chiRNA(+67) and chiRNA(+85) were able to mediate significant cleavage at the two PVALB protospacers (FIGS. **18***c* and **19***b*).

[0235] For all five targets in the EMX1 and PVALB loci, a consistent increase in genome modification efficiency with increasing tracr sequence length was observed. Without wishing to be bound by any theory, the secondary structure formed by the 3' end of the tracrRNA may play a role in enhancing the rate of CRISPR complex formation. An illustration of predicted secondary structures for each of the chimeric RNAs used in this example is provided in FIG. **21**. The secondary structure was predicted using RNAfold (http://raa.tbi.univie.ac.at/egi-bin/RNAfold.cgi) using minimum free energy and partition function algorithm. Pseudocolor for each based (reproduced in grayscale) indicates the probability of pairing. Because chiRNAs with longer tracr sequences were able to cleave targets that were not cleaved by native CRISPR crRNA/tracrRNA hybrids, it is possible that chimeric RNA may be loaded onto Cas9 more efficiently than its native hybrid counterpart. To facilitate the application of Cas9 for site-specific genome editing in eukaryotic cells and organisms, all predicted unique target sites for the *S. pyogenes* Cas9 were computationally identified in the human, mouse, rat, zebra fish, *C. elegans*, and *D. melanogaster* genomes. Chimeric RNAs can be designed for Cas9 enzymes from other microbes to expand the target space of CRISPR RNA-programmable nucleases.

[0236] FIG. **22** illustrates an exemplary bicistronic expression vector for expression of chimeric RNA including up to the +85 nucleotide of wild-type tracr RNA sequence, and SpCas9 with nuclear localization sequences. SpCas9 is expressed from a CBh promoter and terminated with the bGH polyA signal (bGH pA). The expanded sequence illustrated immediately below the schematic corresponds to the region surrounding the guide sequence insertion site, and includes, from 5' to 3',3'-portion of the U6 promoter (first shaded region), BbsI cleavage sites (arrows), partial direct repeat (tracr mate sequence GTTTTAGAGCTA (SEQ ID NO: 27), underlined), loop sequence GAAA, and +85 tracr sequence (underlined sequence following loop sequence). An exemplary guide sequence insert is illustrated below the guide sequence insertion site, with nucleotides of the guide sequence for a selected target represented by an "N".

[0237] Sequences described in the above examples are as follows (polynucleotide sequences are 5' to 3'):

U6-short tracrRNA (*Streptococcus pyogenes* SF370):

(SEQ ID NO: 40)

GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCT

GTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAA

TACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTT

AAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTAT

-continued

ATATCTTGTGGAAAGGACGAAACACC**GGAACCATTCAAAACAGCATAGCAAGTTA**

AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT

TTT (bold = tracrRNA sequence; underline = terminator sequence)

U6-long tracrRNA (*Streptococcus pyogenes* SF370):

(SEQ ID NO: 41)

GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCT

GTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAA

TACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTT

AAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTAT

ATATCTTGTGGAAAGGACGAAACACCGGTAGTATTAAGTATTGTTTTATGGCTGATA

AATTTCTTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTTG

GAACCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA

AAAAGTGGCACCGAGTCGGTGCTTTTTTT

U6-DR-BbsI backbone-DR (*Streptococcus pyogenes* SF370):

(SEQ ID NO: 42)

GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCT

GTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAA

TACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTT

AAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTAT

ATATCTTGTGGAAAGGACGAAACACCGGGTTTTAGAGCTATGCTGTTTTGAATGGTC

CCAAAACGGGTCTTCGAGAAGACGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAA

AC

U6-chimeric RNA-BbsI backbone (*Streptococcus pyogenes* SF370)

(SEQ ID NO: 43)

GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCT

GTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAA

TACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTT

AAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTAT

ATATCTTGTGGAAAGGACGAAACACCGGGTCTTCGAGAAGACCTGTTTTAGAGCTA

GAAATAGCAAGTTAAAATAAGGCTAGTCCG

NLS-SpCas9-EGFP:

(SEQ ID NO: 44)

MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAADKKYSIGLDI

GTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARR

RYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHE

KYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQ

TYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPN

FKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNT

EITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQ

EEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFY

PFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQS

FIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIV

DLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDN

-continued

EENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLIN

GIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLA

GSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEG

IKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSF

LKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAE

RGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVS

DFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMI

AKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATV

RKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYS

VLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYS

LFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVE

QHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAP

AAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDAAAVSKGEELFTG

VVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQ

CFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGI

DFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNT

PIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

SpCas9-EGFP-NLS:

(SEQ ID NO: 45)

MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLF

DSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKK

HERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEG

DLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGE

KKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFL

AAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFF

DQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPH

QIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETI

TPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTE

GMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASL

GTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQL

KRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKA

QVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTT

QKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQEL

DINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQL

LNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDE

NDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKL

ESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIET

NGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKK

DWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLE

AKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHY

-continued

EKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPI

REQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQ

LGGDAAAVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGK

LPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRA

EVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRH

NIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAG

ITLGMDELYKKRPAATKKAGQAKKKK

NLS-SpCas9-EGFP-NLS:

(SEQ ID NO: 46)

MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAADKKYSIGLDI

GTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARR

RYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHE

KYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQ

TYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPN

FKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNT

EITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQ

EEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFY

PFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQS

FIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIV

DLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDN

EENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLIN

GIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLA

GSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEG

IKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSF

LKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAE

RGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVS

DFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMI

AKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATV

RKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYS

VLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYS

LFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVE

QHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAP

AAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDAAAVSKGEELFTG

VVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQ

CFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGI

DFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNT

PIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKKRPAATK

KAGQAKKKK

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NLS-SpCas9-NLS:

(SEQ ID NO: 47)

MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAADKKYSIGLDI

GTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARR

RYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHE

KYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQ

TYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPN

FKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNT

EITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQ

EEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFY

PFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQS

FIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIV

DLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDN

EENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLIN

GIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLA

GSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEG

IKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSF

LKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAE

RGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVS

DFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMI

AKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATV

RKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYS

VLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYS

LFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVE

QHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAP

AAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDKRPAATKKAGQAK

KKK

NLS-mCherry-SpRNase3:

(SEQ ID NO: 48)

MFLFLSLTSFLSSSRTLVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGE

GEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGF

KWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSE

RMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNE

DYTIVEQYERAEGRHSTGGMDELYKGSKQLEELLSTSFDIQFNDLTLLETAFTHTSYANE

HRLLNVSHNERLEFLGDAVLQLIISEYLFAKYPKKTEGDMSKLRSMIVREESLAGFSRFC

SFDAYIKLGKGEEKSGGRRRDTILGDLFEAFLGALLLDKGIDAVRRFLKQVMIPQVEKG

NFERVKDYKTCLQEFLQTKGDVAIDYQVISEKGPAHAKQFEVSIVVNGAVLSKGLGKSK

KLAEQDAAKNALAQLSEV

SpRNase3-mCherry-NLS:

(SEQ ID NO: 49)

MKQLEELLSTSFDIQFNDLTLLETAFTHTSYANEHRLLNVSHNERLEFLGDAV

LQLLISEYLFAKYPKKTEGDMSKLRSMIVREESLAGFSRFCSFDAYIKLGKGEEKSGGRR

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RDTILGDLFEAFLGALLLDKGIDAVRRFLKQVMIPQVEKGNFERVKDYKTCLQEFLQTK

GDVAIDYQVISEKGPAHAKQFEVSIVVNGAVLSKGLGKSKKLAEQDAAKNALAQLSEV

GSVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGP

LPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDS

SLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKD

GGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMD

ELYKKRPAATKKAGQAKKKK

NLS-SpCas9n-NLS (the D10A nickase mutation is lowercase):

(SEQ ID NO: 50)

MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAADKKYSIGLaI

GTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARR

RYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHE

KYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQ

TYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPN

FKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNT

EITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQ

EEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFY

PFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQS

FIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIV

DLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDN

EENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLIN

GIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLA

GSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEG

IKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSF

LKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAE

RGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVS

DFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMI

AKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATV

RKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYS

VLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYS

LFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVE

QHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAP

AAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDKRPAATKKAGQAK

KKK

hEMX1-HR Template-HindII-NheI:

(SEQ ID NO: 51)

GAATGCTGCCCTCAGACCCGCTTCCTCCCTGTCCTTGTCTGTCCAAGGAGA

ATGAGGTCTCACTGGTGGATTTCGGACTACCCTGAGGAGCTGGCACCTGAGGGACA

AGGCCCCCCACCTGCCCAGCTCCAGCCTCTGATGAGGGGTGGGAGAGAGCTACATG

AGGTTGCTAAGAAAGCCTCCCCTGAAGGAGACCACACAGTGTGTGAGGTTGGAGTC

TCTAGCAGCGGGTTCTGTGCCCCCAGGGATAGTCTGGCTGTCCAGGCACTGCTCTTG

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ATATAAACACCACCTCCTAGTTATGAAACCATGCCCATTCTGCCTCTCTGTATGGAA

AAGAGCATGGGGCTGGCCCGTGGGGTGGTGTCCACTTTAGGCCCTGTGGGAGATCA

TGGGAACCCACGCAGTGGGTCATAGGCTCTCTCATTTACTACTCACATCCACTCTGT

GAAGAAGCGATTATGATCTCTCCTCTAGAAACTCGTAGAGTCCCATGTCTGCCGGCT

TCCAGAGCCTGCACTCCTCCACCTTGGCTTGGCTTTGCTGGGGCTAGAGGAGCTAGG

ATGCACAGCAGCTCTGTGACCCTTTGTTTGAGAGGAACAGGAAAACCACCCTTCTCT

CTGGCCCACTGTGTCCTCTTCCTGCCCTGCCATCCCCTTCTGTGAATGTTAGACCCAT

GGGAGCAGCTGGTCAGAGGGGACCCCGGCCTGGGGCCCCTAACCCTATGTAGCCTC

AGTCTTCCCATCAGGCTCTCAGCTCAGCCTGAGTGTTGAGGCCCCAGTGGCTGCTCT

GGGGGCCTCCTGAGTTTCTCATCTGTGCCCCTCCCTCCCTGGCCCAGGTGAAGGTGT

GGTTCCAGAACCGGAGGACAAAGTACAAACGGCAGAAGCTGGAGGAGGAAGGGCC

TGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCA

CGAAGCAGGCCAATGGGGAGGACATCGATGTCACCTCCAATGACaagcttgctagcGGTGG

GCAACCACAAACCCACGAGGGCAGAGTGCTGCTTGCTGCTGGCCAGGCCCCTGCGT

GGGCCCAAGCTGGACTCTGGCCACTCCCTGGCCAGGCTTTGGGGAGGCCTGGAGTC

ATGGCCCCACAGGGCTTGAAGCCCGGGGCCGCCATTGACAGAGGGACAAGCAATGG

GCTGGCTGAGGCCTGGGACCACTTGGCCTTCTCCTCGGAGAGCCTGCCTGCCTGGGC

GGGCCCGCCCGCCACCGCAGCCTCCCAGCTGCTCTCCGTGTCTCCAATCTCCCTTTTG

TTTTGATGCATTTCTGTTTTAATTTATTTTCCAGGCACCACTGTAGTTTAGTGATCCCC

AGTGTCCCCCTTCCCTATGGGAATAATAAAAGTCTCTCTCTTAATGACACGGGCATC

CAGCTCCAGCCCCAGAGCCTGGGGTGGTAGATTCCGGCTCTGAGGGCCAGTGGGGG

CTGGTAGAGCAAACGCGTTCAGGGCCTGGGAGCCTGGGGTGGGGTACTGGTGGAGG

GGGTCAAGGGTAATTCATTAACTCCTCTCTTTTGTTGGGGGACCCTGGTCTCTACCTC

CAGCTCCACAGCAGGAGAAACAGGCTAGACATAGGGAAGGGCCATCCTGTATCTTG

AGGGAGGACAGGCCCAGGTCTTTCTTAACGTATTGAGAGGTGGGAATCAGGCCCAG

GTAGTTCAATGGGAGAGGGAGAGTGCTTCCCTCTGCCTAGAGACTCTGGTGGCTTCT

CCAGTTGAGGAGAAACCAGAGGAAAGGGGAGGATTGGGGTCTGGGGGAGGGAACA

CCATTCACAAAGGCTGACGGTTCCAGTCCGAAGTCGTGGGCCCACCAGGATGCTCA

CCTGTCCTTGGAGAACCGCTGGGCAGGTTGAGACTGCAGAGACAGGGCTTAAGGCT

GAGCCTGCAACCAGTCCCCAGTGACTCAGGGCCTCCTCAGCCCAAGAAAGAGCAAC

GTGCCAGGGCCCGCTGAGCTCTTGTGTTCACCTG

NLS-StCsn1-NLS:

(SEQ ID NO: 52)

MKRPAATKKAGQAKKKKSDLVLGLDIGIGSVGVGILNKVTGEIIHKNSRIFPA

AQAENNLVRRTNRQGRRLARRKKHRRVRLNRLFEESGLITDFTKISINLNPYQLRVKGL

TDELSNEELFIALKNMVKHRGISYLDDASDDGNSSVGDYAQIVKENSKQLETKTPGQIQL

ERYQTYGQLRGDFTVEKDGKKHRLINVFPTSAYRSEALRILQTQQEFNPQITDEFINRYL

EILTGKRKYYHGPGNEKSRTDYGRYRTSGETLDNIFGILIGKCTFYPDEFRAAKASYTAQ

EFNLLNDLNNLTVPTETKKLSKEQKNQIINYVKNEKAMGPAKLFKYIAKLLSCDVADIK

GYRIDKSGKAEIHTFEAYRKMKTLETLDIEQMDRETLDKLAYVLTLNTEREGIQEALEHE

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FADGSFSQKQVDELVQFRKANSSIFGKGWHNFSVKLMMELIPELYETSEEQMTILTRLG

KQKTTSSSNKTKYIDEKLLTEEIYNPVVAKSVRQAIKIVNAAIKEYGDFDNIVIEMARETN

EDDEKKAIQKIQKANKDEKDAAMLKAANQYNGKAELPHSVFHGHKQLATKIRLWHQQ

GERCLYTGKTISIHDLINNSNQFEVDHILPLSITFDDSLANKVLVYATANQEKGQRTPYQ

ALDSMDDAWSFRELKAFVRESKTLSNKKKEYLLTEEDISKFDVRKKFIERNLVDTRYAS

RVVLNALQEHFRAHKIDTKVSVVRGQFTSQLRRHWGIEKTRDTYHHHAVDALIIAASSQ

LNLWKKQKNTLVSYSEDQLLDIETGELISDDEYKESVFKAPYQHFVDTLKSKEFEDSILF

SYQVDSKFNRKISDATIYATRQAKVGKDKADETYVLGKIKDIYTQDGYDAFMKIYKKD

KSKFLMYRHDPQTFEKVIEPILENYPNKQINEKGKEVPCNPFLKYKEEHGYIRKYSKKGN

GPEIKSLKYYDSKLGNHIDITPKDSNNKVVLQSVSPWRADVYFNKTTGKYEILGLKYAD

LQFEKGTGTYKISQEKYNDIKKKEGVDSDSEFKFTLYKNDLLLVKDTETKEQQLFRFLSR

TMPKQKHYVELKPYDKQKFEGGEALIKVLGNVANSGQCKKGLGKSNISIYKVRTDVLG

NQHIIKNEGDKPKLDFKRPAATKKAGQAKKKK

U6-St_tracrRNA(7-97):

(SEQ ID NO: 53)

GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCT

GTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAA

TACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTT

AAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTAT

ATATCTTGTGGAAAGGACGAAACACCGTTACTTAAATCTTGCAGAAGCTACAAAGA

TAAGGCTTCATGCCGAAATCAACACCCTGTCATTTTATGGCAGGGTGTTTTCGTTATT

TAA

U6-DR-spacer-DR (*S. pyogenes* SF370)

(SEQ ID NO: 54)

gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataattggaattaatttgactgtaaa

cacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgc

ttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaacaccgggttttagagctatgctgttttgaatggtccc

aaaacNNNNNNNNNNNNNNNNNNNNNNNNNNNNNgttttagagctatgctgttttgaatggtcccaaaac**T**

TTTTTT (lowercase underline = direct repeat; N = guide sequence; bold = terminator)

Chimeric RNA containing +48 tracr RNA (*S. pyogenes* SF370)

(SEQ ID NO: 55)

gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataattggaattaatttgactgtaaa

cacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgc

ttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaacaccNNNNNNNNNNNNNNNNNN

NNgttttagagctagaaatagcaagttaaaataaggctagtccg**TTTTTTT** (N = guide sequence; first underline = tracr

mate sequence; second underline = tracr sequence; bold = terminator)

Chimeric RNA containing +54 tracr RNA (*S. pyogenes* SF370)

(SEQ ID NO: 56)

gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataattggaattaatttgactgtaaa

cacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgc

ttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaacaccNNNNNNNNNNNNNNNNNN

NNgttttagagctagaaatagcaagttaaaataaggctagtccgttatca**TTTTTTTT** (N = guide sequence; first underline =

tracr mate sequence; second underline = tracr sequence; bold = terminator)

-continued

Chimeric RNA containing +67 tracr RNA (*S. pyogenes* SF370)

(SEQ ID NO: 57)

gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataattggaattaatttgactgtaaa

cacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgc

ttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaacaccNNNNNNNNNNNNNNNNNN

NNgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtg**TTTTTTT**

(N = guide sequence; first underline = tracr mate sequence; second underline = tracr sequence;

bold = terminator)

Chimeric RNA containing +85 tracr RNA (*S. pyogenes* SF370)

(SEQ ID NO: 58)

gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataattggaattaatttgactgtaaa

cacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgc

ttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaacaccNNNNNNNNNNNNNNNNNN

NNgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcTTTTTTT

(N = guide sequence; first underline = tracr mate sequence; second underline =

tracr sequence; bold = terminator)

CBh-NLS-SpCas9-NLS

(SEQ ID NO: 59)

CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACC

CCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTT

TCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATC

AAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCG

CCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTA

CGTATTAGTCATCGCTATTACCATGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTC

CCCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATTTATTTTTTAATTATTTTG

TGCAGCGATGGGGGCGGGGGGGGGGGGGGGCGCGCGCCAGGCGGGGCGGGGCGG

GGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCG

GCGCGCTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGCCCTATAAA

AAGCGAAGCGCGCGGCGGGCGGGAGTCGCTGCGACGCTGCCTTCGCCCCGTGCCCC

GCTCCGCCGCCGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGTTACTCCCAC

AGGTGAGCGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATTAGCTGAGCAAGAGG

TAAGGGTTTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATTACCTGGAGCAC

CTGCCTGAAATCACTTTTTTTCAGGTTGGaccggtgccaccATGGACTATAAGGACCACGA

CGGAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATAAGATGGCCC

CAAAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCCCAGCAGCCGACAAGAAGTA

CAGCATCGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTGATCACCGACG

AGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGGCACAGC

ATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAGGC

CACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATC

TGCTATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTC

CACAGACTGGAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCC

CATCTTCGGCAACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCCACCATCT

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ACCACCTGAGAAAGAAACTGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGATC

TATCTGGCCCTGGCCCACATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGAC

CTGAACCCCGACAACAGCGACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTA

CAACCAGCTGTTCGAGGAAAACCCCATCAACGCCAGCGGCGTGGACGCCAAGGCCA

TCCTGTCTGCCAGACTGAGCAAGAGCAGACGGCTGGAAAATCTGATCGCCCAGCTG

CCCGGCGAGAAGAAGAATGGCCTGTTCGGCAACCTGATTGCCCTGAGCCTGGGCCT

GACCCCCAACTTCAAGAGCAACTTCGACCTGGCCGAGGATGCCAAACTGCAGCTGA

GCAAGGACACCTACGACGACGACCTGGACAACCTGCTGGCCCAGATCGGCGACCAG

TACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATCCTGCTGAGCGAC

ATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCTATGATCAA

GAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCGTGCGGCAGC

AGCTGCCTGAGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCC

GGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCAT

CCTGGAAAAGATGGACGGCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGAC

CTGCTGCGGAAGCAGCGGACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCT

GGGAGAGCTGCACGCCATTCTGCGGCGGCAGGAAGATTTTTACCCATTCCTGAAGG

ACAACCGGGAAAAGATCGAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGC

CCTCTGGCCAGGGGAAACAGCAGATTCGCCTGGATGACCAGAAAGAGCGAGGAAA

CCATCACCCCCTGGAACTTCGAGGAAGTGGTGGACAAGGGCGCTTCCGCCCAGAGC

TTCATCGAGCGGATGACCAACTTCGATAAGAACCTGCCCAACGAGAAGGTGCTGCC

CAAGCACAGCCTGCTGTACGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGA

AATACGTGACCGAGGGAATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAA

GGCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGA

AAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATCTCCGGCGTG

GAAGATCGGTTCAACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATTATCAAG

GACAAGGACTTCCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTGCT

GACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCTATG

CCCACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGC

TGGGGCAGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAA

GACAATCCTGGATTTCCTGAAGTCCGACGGCTTCGCCAACAGAAACTTCATGCAGCT

GATCCACGACGACAGCCTGACCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCG

GCCAGGGCGATAGCCTGCACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATT

AAGAAGGGCATCCTGCAGACAGTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGG

CCGGCACAAGCCCGAGAACATCGTGATCGAAATGGCCAGAGAGAACCAGACCACCC

AGAAGGGACAGAAGAACAGCCGCGAGAGAATGAAGCGGATCGAAGAGGGCATCAA

AGAGCTGGGCAGCCAGATCCTGAAAGAACACCCCGTGGAAAACACCCAGCTGCAGA

ACGAGAAGCTGTACCTGTACTACCTGCAGAATGGGCGGGATATGTACGTGGACCAG

GAACTGGACATCAACCGGCTGTCCGACTACGATGTGGACCATATCGTGCCTCAGAG

CTTTCTGAAGGACGACTCCATCGACAACAAGGTGCTGACCAGAAGCGACAAGAACC

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GGGGCAAGAGCGACAACGTGCCCTCCGAAGAGGTCGTGAAGAAGATGAAGAACTA

CTGGCGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTGA

CCAAGGCCGAGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAG

ACAGCTGGTGGAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCC

GGATGAACACTAAGTACGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATC

ACCCTGAAGTCCAAGCTGGTGTCCGATTTCCGGAAGGATTTCCAGTTTTACAAAGTG

CGCGAGATCAACAACTACCACCACGCCCACGACGCCTACCTGAACGCCGTCGTGGG

AACCGCCCTGATCAAAAAGTACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACT

ACAAGGTGTACGACGTGCGGAAGATGATCGCCAAGAGCGAGCAGGAAATCGGCAA

GGCTACCGCCAAGTACTTCTTCTACAGCAACATCATGAACTTTTTCAAGACCGAGAT

TACCCTGGCCAACGGCGAGATCCGGAAGCGGCCTCTGATCGAGACAAACGGCGAAA

CCGGGGAGATCGTGTGGGATAAGGGCCGGGATTTTGCCACCGTGCGGAAAGTGCTG

AGCATGCCCCAAGTGAATATCGTGAAAAAGACCGAGGTGCAGACAGGCGGCTTCAG

CAAAGAGTCTATCCTGCCCAAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGG

ACTGGGACCCTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGTGC

TGGTGGTGGCCAAAGTGGAAAAGGGCAAGTCCAAGAAACTGAAGAGTGTGAAAGA

GCTGCTGGGGATCACCATCATGGAAAGAAGCAGCTTCGAGAAGAATCCCATCGACT

TTCTGGAAGCCAAGGGCTACAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCCT

AAGTACTCCCTGTTCGAGCTGGAAAACGGCCGGAAGAGAATGCTGGCCTCTGCCGG

CGAACTGCAGAAGGGAAACGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGT

ACCTGGCCAGCCACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAA

CAGCTGTTTGTGGAACAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAG

CGAGTTCTCCAAGAGAGTGATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGC

CTACAACAAGCACCGGGATAAGCCCATCAGAGAGCAGGCCGAGAATATCATCCACC

TGTTTACCCTGACCAATCTGGGAGCCCCTGCCGCCTTCAAGTACTTTGACACCACCA

TCGACCGGAAGAGGTACACCAGCACCAAAGAGGTGCTGGACGCCACCCTGATCCAC

CAGAGCATCACCGGCCTGTACGAGACACGGATCGACCTGTCTCAGCTGGGAGGCGA

CTTTCTTTTTCTTAGCTTGACCAGCTTTCTTAGTAGCAGGACGCTTTAA

(underline = NLS-hSpCas9-NLS)

[0238] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR1Cas9 (with PAM of NNAGAAW)

[0239] NNNNNNNNNNNNNNNNNNN gtttttgtactctcaagatttaGAAAtaaatcttgcagaagctacaaagataaggct tcatgccgaaatcaacaccctgtcatttttatggcagggtgattcgttatttaaTTTT TT (SEQ ID NO: 21) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0240] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR1 Cas9 (with PAM of NNAGAAW)

[0241] NNNNNNNNNNNNNNNNNN gtttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccgaaat caacaccctgtcatttttatggcagggtgttttcgttatttaaTTTTTT (SEQ ID NO: 22) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0242] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR1 Cas9 (with PAM of NNAGAAW)

[0243] NNNNNNNNNNNNNNNNNNN gtttttgtactctcaGAAAtgcagaagctacaaagataaggcttcaccgaaatca acaccctgtcatttttatggcagggtgtTTTTTT (SEQ ID NO: 23) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0244] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR1 Cas9 (with PAM of NNAGAAW)

[0245] NNNNNNNNNNNNNNNNNNN gttattgtactctcaagatttaGAAAtaaatcttgcagaagctacaaagataagg cttcatgccgaaatcaacaccctgtcatttttatggcagggtgttttcgttatttaaTTT TTT (SEQ ID NO: 60) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0246] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR1 Cas9 (with PAM of NNAGAAW)

[0247] NNNNNNNNNNNNNNNNNNN gttattgtactacaGAAAtgcagaagctacaaagataaggcttcatg ccgaaatcaacaccctgtcatttttatggcagggtgttttcgttatttaaTTTTTT (SEQ ID NO: 61) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0248] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR1 Cas9 (with PAM of NNAGAAW)

[0249] NNNNNNNNNNNNNNNNNNN gttattgtactctcaGAAAtgcagaagctacaaagataaggcttcatg ccgaaatcaacaccctgtcatttttatggcagggtgtTTTTTT (SEQ ID NO: 62) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0250] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR1 Cas9 (with PAM of NNAGAAW)

[0251] NNNNNNNNNNNNNNNNNNN gttattgtactctcaagatttaGAAAtaaatcttgcagaagctacaatgataagg atcatgccgaaatcaacaccctgtcatttttatggcagggtgtttttcgttatttaaTTT TTT (SEQ ID NO: 63) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0252] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR1 Cas9 (with PAM of NNAGAAW)

[0253] NNNNNNNNNNNNNNNNNNN gttattgtactctcaGAAAtgcagaagctacaatgataaggcttcatgc cgaaatcaacaccctgtcatttttatggcagggtgttttcgttatttaaTTTTTT (SEQ ID NO: 64) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0254] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR1 Cas9 (with PAM of NNAGAAW)

[0255] NNNNNNNNNNNNNNNNNNN gttattgtactctcaGAAAtgcagaagctacaatgataaggcttcatg ccgaaatcaacaccctgtcatttttatggcagggtgtTTTTTT (SEQ ID NO: 65) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0256] Example chimeric RNA for *S. thermophilus* LMD-9 CRISPR3 Cas9 (with PAM of NGGNG)

[0257] NNNNNNNNNNNNNNNNNNN gttttagagctgtgGAAAcacagcgagttaaaataaggcttagtccgtactc aacttgaaaaggtggcaccgattcggtgtTTTTTT (SEQ ID NO: 66) (N=guide sequence; first underline=tracr mate sequence; second underline=tracr sequence; bold=terminator)

[0258] Codon-optimized version of Cas9 from *S. thermophilus* LMD-9 CRISPR3 locus (with an NLS at both 5' and 3' ends)

(SEQ ID NO: 67)

```
ATGAAAAGGCCGGCGGCCACGAAAAAGGCCGGCCAGGCAAAAAAGAAAAA

GACCAAGCCCTACAGCATCGGCCTGGACATCGGCACCAATAGCGTGGGCT

GGGCCGTGACCACCGACAACTACAAGGTGCCCAGCAAGAAAATGAAGGTG

CTGGGCAACACCTCCAAGAAGTACATCAAGAAAAACCTGCTGGGCGTGCT

GCTGTTCGACAGCGGCATTACAGCCGAGGGCAGACGGCTGAAGAGAACCG

CCAGACGGCGGTACACCCGGCGGAGAAACAGAATCCTGTATCTGCAAGAG

ATCTTCAGCACCGAGATGGCTACCCTGGACGACGCCTTCTTCCAGCGGCT

GGACGACAGCTTCCTGGTGCCCGACGACAAGCGGGACAGCAAGTACCCCA
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TCTTCGGCAACCTGGTGGAAGAGAAGGCCTACCACGACGAGTTCCCCACC

ATCTACCACCTGAGAAAGTACCTGGCCGACAGCACCAAGAAGGCCGACCT

GAGACTGGTGTATCTGGCCCTGGCCCACATGATCAAGTACCGGGGCCACT

TCCTGATCGAGGGCGAGTTCAACAGCAAGAACAACGACATCCAGAAGAAC

TTCCAGGACTTCCTGGACACCTACAACGCCATCTTCGAGAGCGACCTGTC

CCTGGAAAACAGCAAGCAGCTGGAAGAGATCGTGAAGGACAAGATCAGCA

AGCTGGAAAAGAAGGACCGCATCCTGAAGCTGTTCCCCGGCGAGAAGAAC

AGCGGAATCTTCAGCGAGTTTCTGAAGCTGATCGTGGGCAACCAGGCCGA

CTTCAGAAAGTGCTTCAACCTGGACGAGAAAGCCAGCCTGCACTTCAGCA

AAGAGAGCTACGACGAGGACCTGGAAACCCTGCTGGGATATATCGGCGAC

GACTACAGCGACGTGTTCCTGAAGGCCAAGAAGCTGTACGACGCTATCCT

GCTGAGCGGCTTCCTGACCGTGACCGACAACGAGACAGAGGCCCCACTGA

GCAGCGCCATGATTAAGCGGTACAACGAGCACAAAGAGGATCTGGCTCTG

CTGAAAGAGTACATCCGGAACATCAGCCTGAAAACCTACAATGAGGTGTT

CAAGGACGACACCAAGAACGGCTACGCCGGCTACATCGACGGCAAGACCA

ACCAGGAAGATTTCTATGTGTACCTGAAGAAGCTGCTGGCCGAGTTCGAG

GGGGCCGACTACTTTCTGGAAAAAATCGACCGCGAGGATTTCCTGCGGAA

GCAGCGGACCTTCGACAACGGCAGCATCCCCTACCAGATCCATCTGCAGG

AAATGCGGGCCATCCTGGACAAGCAGGCCAAGTTCTACCCATTCCTGGCC

AAGAACAAAGAGCGGATCGAGAAGATCCTGACCTTCCGCATCCCTTACTA

CGTGGGCCCCCTGGCCAGAGGCAACAGCGATTTTGCCTGGTCCATCCGGA

AGCGCAATGAGAAGATCACCCCCTGGAACTTCGAGGACGTGATCGACAAA

GAGTCCAGCGCCGAGGCCTTCATCAACCGGATGACCAGCTTCGACCTGTA

CCTGCCCGAGGAAAAGGTGCTGCCCAAGCACAGCCTGCTGTACGAGACAT

TCAATGTGTATAACGAGCTGACCAAAGTGCGGTTTATCGCCGAGTCTATG

CGGGACTACCAGTTCCTGGACTCCAAGCAGAAAAAGGACATCGTGCGGCT

GTACTTCAAGGACAAGCGGAAAGTGACCGATAAGGACATCATCGAGTACC

TGCACGCCATCTACGGCTACGATGGCATCGAGCTGAAGGGCATCGAGAAG

CAGTTCAACTCCAGCCTGAGCACATACCACGACCTGCTGAACATTATCAA

CGACAAAGAATTTCTGGACGACTCCAGCAACGAGGCCATCATCGAAGAGA

TCATCCACACCCTGACCATCTTTGAGGACCGCGAGATGATCAAGCAGCGG

CTGAGCAAGTTCGAGAACATCTTCGACAAGAGCGTGCTGAAAAAGCTGAG

CAGACGGCACTACACCGGCTGGGGCAAGCTGAGCGCCAAGCTGATCAACG

GCATCCGGGACGAGAAGTCCGGCAACACAATCCTGGACTACCTGATCGAC

GACGGCATCAGCAACCGGAACTTCATGCAGCTGATCCACGACGACGCCCT

GAGCTTCAAGAAGAAGATCCAGAAGGCCCAGATCATCGGGGACGAGGACA

AGGGCAACATCAAAGAAGTCGTGAAGTCCCTGCCCGGCAGCCCCGCCATC

AAGAAGGGAATCCTGCAGAGCATCAAGATCGTGGACGAGCTCGTGAAAGT

GATGGGCGGCAGAAAGCCCGAGAGCATCGTGGTGGAAATGGCTAGAGAGA
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ACCAGTACACCAATCAGGGCAAGAGCAACAGCCAGCAGAGACTGAAGAGA

CTGGAAAAGTCCCTGAAAGAGCTGGGCAGCAAGATTCTGAAAGAGAATAT

CCCTGCCAAGCTGTCCAAGATCGACAACAACGCCCTGCAGAACGACCGGC

TGTACCTGTACTACCTGCAGAATGGCAAGGACATGTATACAGGCGACGAC

CTGGATATCGACCGCCTGAGCAACTACGACATCGACCATATTATCCCCCA

GGCCTTCCTGAAAGACAACAGCATTGACAACAAAGTGCTGGTGTCCTCCG

CCAGCAACCGCGGCAAGTCCGATGATGTGCCCAGCCTGGAAGTCGTGAAA

AAGAGAAAGACCTTCTGGTATCAGCTGCTGAAAAGCAAGCTGATTAGCCA

GAGGAAGTTCGACAACCTGACCAAGGCCGAGAGAGGCGGCCTGAGCCCTG

AAGATAAGGCCGGCTTCATCCAGAGACAGCTGGTGGAAACCCGGCAGATC

ACCAAGCACGTGGCCAGACTGCTGGATGAGAAGTTTAACAACAAGAAGGA

CGAGAACAACCGGGCCGTGCGGACCGTGAAGATCATCACCCTGAAGTCCA

CCCTGGTGTCCCAGTTCCGGAAGGACTTCGAGCTGTATAAAGTGCGCGAG

ATCAATGACTTTCACCACGCCCACGACGCCTACCTGAATGCCGTGGTGGC

TTCCGCCCTGCTGAAGAAGTACCCTAAGCTGGAACCCGAGTTCGTGTACG

GCGACTACCCCAAGTACAACTCCTTCAGAGAGCGGAAGTCCGCCACCGAG

AAGGTGTACTTCTACTCCAACATCATGAATATCTTTAAGAAGTCCATCTC

CCTGGCCGATGGCAGAGTGATCGAGCGGCCCCTGATCGAAGTGAACGAAG

AGACAGGCGAGAGCGTGTGGAACAAAGAAAGCGACCTGGCCACCGTGCGG

CGGGTGCTGAGTTATCCTCAAGTGAATGTCGTGAAGAAGGTGGAAGAACA

GAACCACGGCCTGGATCGGGGCAAGCCCAAGGGCCTGTTCAACGCCAACC

TGTCCAGCAAGCCTAAGCCCAACTCCAACGAGAATCTCGTGGGGGCCAAA

GAGTACCTGGACCCTAAGAAGTACGGCGGATACGCCGGCATCTCCAATAG

CTTCACCGTGCTCGTGAAGGGCACAATCGAGAAGGGCGCTAAGAAAAAGA

TCACAAACGTGCTGGAATTTCAGGGGATCTCTATCCTGGACCGGATCAAC

TACCGGAAGGATAAGCTGAACTTTCTGCTGGAAAAAGGCTACAAGGACAT

TGAGCTGATTATCGAGCTGCCTAAGTACTCCCTGTTCGAACTGAGCGACG

GCTCCAGACGGATGCTGGCCTCCATCCTGTCCACCAACAACAAGCGGGGC

GAGATCCACAAGGGAAACCAGATCTTCCTGAGCCAGAAATTTGTGAAACT

GCTGTACCACGCCAAGCGGATCTCCAACACCATCAATGAGAACCACCGGA

AATACGTGGAAAACCACAAGAAAGAGTTTGAGGAACTGTTCTACTACATC

CTGGAGTTCAACGAGAACTATGTGGGAGCCAAGAAGAACGGCAAACTGCT

GAACTCCGCCTTCCAGAGCTGGCAGAACCACAGCATCGACGAGCTGTGCA

GCTCCTTCATCGGCCCTACCGGCAGCGAGCGGAAGGGACTGTTTGAGCTG

ACCTCCAGAGGCTCTGCCGCCGACTTTGAGTTCCTGGGAGTGAAGATCCC

CCGGTACAGAGACTACACCCCCTCTAGTCTGCTGAAGGACGCCACCCTGA

TCCACCAGAGCGTGACCGGCCTGTACGAAACCCGGATCGACCTGGCTAAG

CTGGGCGAGGGAAAGCGTCCTGCTGCTACTAAGAAAGCTGGTCAAGCTAA

GAAAAAGAAATAA

Example 5

RNA-Guided Editing of Bacterial Genomes Using CRISPR-Cas Systems

[0259] Applicants used the CRISPR-associated endonuclease Cas9 to introduce precise mutations in the genomes of *Streptococcus pneumoniae* and *Escherichia coli*. The approach relied on Cas9-directed cleavage at the targeted site to kill unmutated cells and circumvented the need for selectable markers or counter-selection systems. Cas9 specificity was reprogrammed by changing the sequence of short CRISPR RNA (crRNA) to make single- and multi-nucleotide changes carried on editing templates. Simultaneous use of two crRNAs enabled multiplex mutagenesis. In *S. pneumoniae*, nearly 100% of cells that survived Cas9 cleavage contained the desired mutation, and 65% when used in combination with recombineering in *E. coli*. Applicants exhaustively analyzed Cas9 target requirements to define the range of targetable sequences and showed strategies for editing sites that do not meet these requirements, suggesting the versatility of this technique for bacterial genome engineering.

[0260] The understanding of gene function depends on the possibility of altering DNA sequences within the cell in a controlled fashion. Site-specific mutagenesis in eukaryotes is achieved by the use of sequence-specific nucleases that promote homologous recombination of a template DNA containing the mutation of interest. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and homing meganucleases can be programmed to cleave genomes in specific locations, but these approaches require engineering of new enzymes for each target sequence. In prokaryotic organisms, mutagenesis methods either introduce a selection marker in the edited locus or require a two-step process that includes a counter-selection system. More recently, phage recombination proteins have been used for recombineering, a technique that promotes homologuous recombination of linear DNA or oligonucleotides. However, because there is no selection of mutations, recombineering efficiency can be relatively low (0.1-10% for point mutations down to 10^{-5}-10^{-6} for larger modifications), in many cases requiring the screening of a large number of colonies. Therefore new technologies that are affordable, easy to use and efficient are still in need for the genetic engineering of both eukaryotic and prokaryotic organisms.

[0261] Recent work on the CRISPR (clustered, regularly interspaced, short palindromic repeats) adaptive immune system of prokaryotes has led to the identification of nucleases whose sequence specificity is programmed by small RNAs. CRISPR loci are composed of a series of repeats separated by 'spacer' sequences that match the genomes of bacteriophages and other mobile genetic elements. The repeat-spacer array is transcribed as a long precursor and processed within repeat sequences to generate small crRNA that specify the target sequences (also known as protospacers) cleaved by CRISPR systems. Essential for cleavage is the presence of a sequence motif immediately downstream of the target region, known as the protospacer-adjacent motif (PAM). CRISPR-associated (cas) genes usually flank the repeat-spacer array and encode the enzymatic machinery responsible for crRNA biogenesis and targeting. Cas9 is a dsDNA endonuclease that uses a crRNA guide to specify the site of cleavage. Loading of the crRNA guide onto Cas9 occurs during the processing of the crRNA precursor and requires a small RNA antisense to the precursor, the tracrRNA, and RNAse III. In contrast to

genome editing with ZFNs or TALENs, changing Cas9 target specificity does not require protein engineering but only the design of the short crRNA guide.

[0262] Applicants recently showed in *S. pneumoniae* that the introduction of a CRISPR system targeting a chromosomal locus leads to the killing of the transformed cells. It was observed that occasional survivors contained mutations in the target region, suggesting that Cas9 dsDNA endonuclease activity against endogenous targets could be used for genome editing. Applicants showed that marker-less mutations can be introduced through the transformation of a template DNA fragment that will recombine in the genome and eliminate Cas9 target recognition. Directing the specificity of Cas9 with several different crRNAs allows for the introduction of multiple mutations at the same time. Applicants also characterized in detail the sequence requirements for Cas9 targeting and show that the approach can be combined with recombineering for genome editing in *E. coli*.

[0263] Results: Genome Editing by Cas9 Cleavage of a Chromosomal Target

[0264] *S. pneumoniae* strain crR6 contains a Cas9-based CRISPR system that cleaves a target sequence present in the bacteriophage ϕ8232.5. This target was integrated into the srtA chromosomal locus of a second strain $R6^{8232.5}$. An altered target sequence containing a mutation in the PAM region was integrated into the srtA locus of a third strain $R6^{370.1}$, rendering this strain 'immune' to CRISPR cleavage (FIG. **28***a*). Applicants transformed $R6^{8232.5}$ and $R6^{370.1}$ cells with genomic DNA from crR6 cells, expecting that successful transformation of $R6^{8232.5}$ cells should lead to cleavage of the target locus and cell death. Contrary to this expectation, Applicants isolated $R6^{8232.5}$ transformants, albeit with approximately 10-fold less efficiency than $R6^{370.1}$ transformants (FIG. **28***b*). Genetic analysis of eight $R6^{8232.5}$ transformants (FIG. **28**) revealed that the great majority are the product of a double recombination event that eliminates the toxicity of Cas9 targeting by replacing the ϕ8232.5 target with the crR6 genome's wild-type srtA locus, which does not contain the protospacer required for Cas9 recognition. These results were proof that the concurrent introduction of a CRISPR system targeting a genomic locus (the targeting construct) together with a template for recombination into the targeted locus (the editing template) led to targeted genome editing (FIG. **23***a*).

[0265] To create a simplified system for genome editing, Applicants modified the CRISPR locus in strain crR6 by deleting cas1, cast and csn2, genes which have been shown to be dispensable for CRISPR targeting, yielding strain crR6M (FIG. **28***a*). This strain retained the same properties of crR6 (FIG. **28***b*). To increase the efficiency of Cas9-based editing and demonstrate that a template DNA of choice can be used to control the mutation introduced, Applicants co-transformed $R6^{8232.5}$ cells with PCR products of the wild-type srtA gene or the mutant $R6^{370.1}$ target, either of which should be resistant to cleavage by Cas9. This resulted in a 5- to 10-fold increase of the frequency of transformation compared with genomic crR6 DNA alone (FIG. **23***b*). The efficiency of editing was also substantially increased, with 8/8 transformants tested containing a wild-type srtA copy and 7/8 containing the PAM mutation present in the $R6^{370.1}$ target (FIG. **23***b* and FIG. **29***a*). Taken together, these results showed the potential of genome editing assisted by Cas9.

[0266] Analysis of Cas9 Target Requirements:

[0267] To introduce specific changes in the genome, one must use an editing template carrying mutations that abolish Cas9-mediated cleavage, thereby preventing cell death. This is easy to achieve when the deletion of the target or its replacement by another sequence (gene insertion) is sought. When the goal is to produce gene fusions or to generate single-nucleotide mutations, the abolishment of Cas9 nuclease activity will only be possible by introducing mutations in the editing template that alter either the PAM or the protospacer sequences. To determine the constraints of CRISPR-mediated editing, Applicants performed an exhaustive analysis of PAM and protospacer mutations that abrogate CRISPR targeting.

[0268] Previous studies proposed that *S. pyogenes* Cas9 requires an NGG PAM immediately downstream of the protospacer. However, because only a very limited number of PAM-inactivating mutations have been described so far, Applicants conducted a systematic analysis to find all 5-nucleotide sequences following the protospacer that eliminate CRISPR cleavage. Applicants used randomized oligonucleotides to generate all possible 1,024 PAM sequences in a heterogeneous PCR product that was transformed into crR6 or R6 cells. Constructs carrying functional PAMs were expected to be recognized and destroyed by Cas9 in crR6 but not R6 cells (FIG. **24***a*). More than 2×10^5 colonies were pooled together to extract DNA for use as template for the co-amplification of all targets. PCR products were deep sequenced and found to contain all 1,024 sequences, with coverage ranging from 5 to 42,472 reads (See section "Analysis of deep sequencing data"). The functionality of each PAM was estimated by the relative proportion of its reads in the crR6 sample over the R6 sample. Analysis of the first three bases of the PAM, averaging over the two last bases, clearly showed that the NGG pattern was under-represented in crR6 transformants (FIG. **24***b*). Furthermore, the next two bases had no detectable effect on the NGG PAM (See section "Analysis of deep sequencing data"), demonstrating that the NGGNN sequence was sufficient to license Cas9 activity. Partial targeting was observed for NAG PAM sequences (FIG. **24***b*). Also the NNGGN pattern partially inactivated CRISPR targeting (Table G), indicating that the NGG motif can still be recognized by Cas9 with reduced efficiency when shifted by 1 bp. These data shed light onto the molecular mechanism of Cas9 target recognition, and they revealed that NGG (or CCN on the complementary strand) sequences are sufficient for Cas9 targeting and that NGG to NAG or NNGGN mutations in the editing template should be avoided. Owing to the high frequency of these tri-nucleotide sequences (once every 8 bp), this means that almost any position of the genome can be edited. Indeed, Applicants tested ten randomly chosen targets carrying various PAMs and all were found to be functional (FIG. **30**).

[0269] Another way to disrupt Cas9-mediated cleavage is to introduce mutations in the protospacer region of the editing template. It is known that point mutations within the 'seed sequence' (the 8 to 10 protospacer nucleotides immediately adjacent to the PAM) can abolish cleavage by CRISPR nucleases. However, the exact length of this region is not known, and it is unclear whether mutations to any nucleotide in the seed can disrupt Cas9 target recognition. Applicants followed the same deep sequencing approach described above to randomize the entire protospacer sequence involved in base pair contacts with the crRNA and to determine all

sequences that disrupt targeting. Each position of the 20 matching nucleotides (14) in the spcl target present in $R6^{8232.5}$ cells (FIG. **23***a*) was randomized and transformed into crR6 and R6 cells (FIG. **24***a*). Consistent with the presence of a seed sequence, only mutations in the 12 nucleotides immediately upstream of the PAM abrogated cleavage by Cas9 (FIG. **24***c*). However, different mutations displayed markedly different effects. The distal (from the PAM) positions of the seed (12 to 7) tolerated most mutations and only one particular base substitution abrogated targeting. In contrast, mutations to any nucleotide in the proximal positions (6 to 1, except 3) eliminated Cas9 activity, although at different levels for each particular substitution. At position 3, only two substitutions affected CRISPR activity and with different strength. Applicants concluded that, although seed sequence mutations can prevent CRISPR targeting, there are restrictions regarding the nucleotide changes that can be made in each position of the seed. Moreover, these restrictions can most likely vary for different spacer sequences. Therefore Applicants believe that mutations in the PAM sequence, if possible, should be the preferred editing strategy. Alternatively, multiple mutations in the seed sequence may be introduced to prevent Cas9 nuclease activity.

[0270] Cas9-Mediated Genome Editing in *S. pneumonia:*

[0271] To develop a rapid and efficient method for targeted genome editing, Applicants engineered strain crR6Rk, a strain in which spacers can be easily introduced by PCR (FIG. **33**). Applicants decided to edit the β-galactosidase (bgaA) gene of *S. pneumoniae*, whose activity can be easily measured. Applicants introduced alanine substitutions of amino acids in the active site of this enzyme: R481A (R→A) and N563A,E564A (NE→AA) mutations. To illustrate different editing strategies, Applicants designed mutations of both the PAM sequence and the protospacer seed. In both cases the same targeting construct with a crRNA complementary to a region of the β-galactosidase gene that is adjacent to a TGG PAM sequence (CCA in the complementary strand, FIG. **26**) was used. The R→A editing template created a three-nucleotide mismatch on the protospacer seed sequence (CGT to GCA, also introducing a BtgZI restriction site). In the NE→AA editing template Applicants simultaneously introduced a synonymous mutation that created an inactive PAM (TGG to TTG) along with mutations that are 218 nt downstream of the protospacer region (AAT GAA to GCT GCA, also generating a TseI restriction site). This last editing strategy demonstrated the possibility of using a remote PAM to make mutations in places where a proper target may be hard to choose. For example, although the *S. pneumoniae* R6 genome, which has a 39.7% GC content, contains on average one PAM motif every 12 bp, some PAM motifs are separated by up to 194 bp (FIG. **33**). In addition Applicants designed a AbgaA in-frame deletion of 6,664 bp. In all three cases, co-transformation of the targeting and editing templates produced 10-times more kanamycin-resistant cells than co-transformation with a control editing template containing wild-type bgaA sequences (FIG. **25***b*). Applicants genotyped 24 transformants (8 for each editing experiment) and found that all but one incorporated the desired change (FIG. **25***c*). DNA sequencing also confirmed not only the presence of the introduced mutations but also the absence of secondary mutations in the target region (FIG. **29***b,c*). Finally, Applicants measured β-galactosidase activity to confirm that all edited cells displayed the expected phenotype (FIG. **25***d*).

[0272] Cas9-mediated editing can also be used to generate multiple mutations for the study of biological pathways. Applicants decided to illustrate this for the sortase-dependent pathway that anchors surface proteins to the envelope of Gram-positive bacteria. Applicants introduced a sortase deletion by co-transformation of a chloramphenicol-resistant targeting construct and a ΔsrtA editing template (FIG. **33***a,b*), followed by a AbgaA deletion using a kanamycin-resistant targeting construct that replaced the previous one. In *S. pneumoniae*, β-galactosidase is covalently linked to the cell wall by sortase. Therefore, deletion of srtA results in the release of the surface protein into the supernatant, whereas the double deletion has no detectable β-galactosidase activity (FIG. **34***c*). Such a sequential selection can be iterated as many times as required to generate multiple mutations.

[0273] These two mutations may also be introduced at the same time. Applicants designed a targeting construct containing two spacers, one matching srtA and the other matching bgaA, and co-transformed it with both editing templates at the same time (FIG. **25***e*). Genetic analysis of transformants showed that editing occurred in 6/8 cases (FIG. **250**. Notably, the remaining two clones each contained either a ΔsrtA or a ΔbgaA deletion, suggesting the possibility of performing combinatorial mutagenesis using Cas9. Finally, to eliminate the CRISPR sequences, Applicants introduced a plasmid containing the bgaA target and a spectinomycin resistance gene along with genomic DNA from the wild-type strain R6. Spectinomycin-resistant transformants that retain the plasmid eliminated the CRISPR sequences (FIG. **34***a,d*).

[0274] Mechanism and Efficiency of Editing:

[0275] To understand the mechanisms underlying genome editing with Cas9, Applicants designed an experiment in which the editing efficiency was measured independently of Cas9 cleavage. Applicants integrated the ermAM erythromycin resistance gene in the srtA locus, and introduced a premature stop codon using Cas9-mediated editing (FIG. **33**). The resulting strain (JEN53) contains an ermAM(stop) allele and is sensitive to erythromycin. This strain may be used to assess the efficiency at which the ermAM gene is repaired by measuring the fraction of cells that restore antibiotic resistance with or without the use of Cas9 cleavage. JEN53 was transformed with an editing template that restores the wild-type allele, together with either a kanamycin-resistant CRISPR construct targeting the ermAM(stop) allele (CRISPR::ermAM(stop)) or a control construct without a spacer (CRISPR::Ø) (FIG. **26***a,b*). In the absence of kanamycin selection, the fraction of edited colonies was on the order of 10^{-2} (erythromycin-resistant cfu/total cfu) (FIG. **26***c*), representing the baseline frequency of recombination without Cas9-mediated selection against unedited cells. However, if kanamycin selection was applied and the control CRISPR construct was co-transformed, the fraction of edited colonies increased to about 10^{-1} (kanamycin- and erythromycin-resistant cfu/kanamycin-resistant cfu) (FIG. **26***c*). This result shows that selection for the recombination of the CRISPR locus co-selected for recombination in the ermAM locus independently of Cas9 cleavage of the genome, suggesting that a subpopulation of cells is more prone to transformation and/or recombination. Transformation of the CRISPR::ermAM(stop) construct followed by kanamycin selection resulted in an increase of the fraction of erythromycin-resistant, edited cells to 99% (FIG. **26***c*). To determine if this increase is caused by the killing of non-edited cells, Applicants compared the kanamycin-resistant colony forming

units (cfu) obtained after co-transformation of JEN53 cells with the CRISPR::ermAM(stop) or CRISPR::Ø constructs.

[0276] Applicants counted 5.3 times less kanamycin-resistant colonies after transformation of the ermAM(stop) construct ($2.5\times10^4/4.7\times10^3$, FIG. **35***a*), a result that suggests that indeed targeting of a chromosomal locus by Cas9 leads to the killing of non-edited cells. Finally, because the introduction of dsDNA breaks in the bacterial chromosome is known to trigger repair mechanisms that increase the rate of recombination of the damaged DNA, Applicants investigated whether cleavage by Cas9 induces recombination of the editing template. Applicants counted 2.2 times more colonies after co-transformation with the CRISPR::erm(stop) construct than with the CRISPR::Ø construct (FIG. **26***d*), indicating that there was a modest induction of recombination. Taken together, these results showed that co-selection of transformable cells, induction of recombination by Cas9-mediated cleavage and selection against non-edited cells, each contributed to the high efficiency of genome editing in *S. pneumoniae.*

[0277] As cleavage of the genome by Cas9 should kill non-edited cells, one would not expect to recover any cells that received the kanamycin resistance-containing Cas9 cassette but not the editing template. However, in the absence of the editing template Applicants recovered many kanamycin-resistant colonies after transformation of the CRISPR::ermAM(stop) construct (FIG. **35***a*). These cells that 'escape' CRISPR-induced death produced a background that determined a limit of the method. This background frequency may be calculated as the ratio of CRISPR::ermAM(stop)/CRISPR::Ø cfu, 2.6×10^{-3} ($7.1\times10^1/2.7\times10^4$) in this experiment, meaning that if the recombination frequency of the editing template is less than this value, CRISPR selection may not efficiently recover the desired mutants above the background. To understand the origin of these cells, Applicants genotyped 8 background colonies and found that 7 contained deletions of the targeting spacer (FIG. **35***b*) and one harbored a presumably inactivating mutation in Cas9 (FIG. **35***c*).

[0278] Genome Editing with Cas9 in *E. coli:*

[0279] The activation of Cas9 targeting through the chromosomal integration of a CRISPR-Cas system is only possible in organisms that are highly recombinogenic. To develop a more general method that is applicable to other microbes, Applicants decided to perform genome editing in *E. coli* using a plasmid-based CRISPR-Cas system. Two plasmids were constructed: a pCas9 plasmid carrying the tracrRNA, Cas9 and a chloramphenicol resistance cassette (FIG. **36**), and a pCRISPR kanamycin-resistant plasmid carrying the array of CRISPR spacers. To measure the efficiency of editing independently of CRISPR selection, Applicants sought to introduce an A to C transversion in the rpsL gene that confers streptomycin resistance. Applicants constructed a pCRISPR::rpsL plasmid harboring a spacer that would guide Cas9 cleavage of the wild-type, but not the mutant rpsL allele (FIG. **27***b*). The pCas9 plasmid was first introduced into *E. coli* MG1655 and the resulting strain was co-transformed with the pCRISPR::rpsL plasmid and W542, an editing oligonucleotide containing the A to C mutation. streptomycin-resistant colonies after transformation of the pCRISPR::rpsL plasmid were only recovered, suggesting that Cas9 cleavage induces recombination of the oligonucleotide (FIG. **37**). However, the number of streptomycin-resistant colonies was two orders of magnitude lower than the number of kanamycin-resistant colonies, which are presumably cells that escape cleavage by Cas9. Therefore, in these conditions, cleavage by Cas9 facilitated the introduction of the mutation, but with an efficiency that was not enough to select the mutant cells above the background of 'escapers'.

[0280] To improve the efficiency of genome editing in *E. coli*, Applicants applied their CRISPR system with recombineering, using Cas9-induced cell death to select for the desired mutations. The pCas9 plasmid was introduced into the recombineering strain HME63 (31), which contains the Gam, Exo and Beta functions of the □-red phage. The resulting strain was co-transformed with the pCRISPR::rpsL plasmid (or a pCRISPR::Ø control) and the W542 oligonucleotide (FIG. **27***a*). The recombineering efficiency was 5.3×10^{-5}, calculated as the fraction of total cells that become streptomycin-resistant when the control plasmid was used (FIG. **27***c*). In contrast, transformation with the pCRISPR::rpsL plasmid increased the percentage of mutant cells to 65±14% (FIGS. **27***c* and **29***f*). Applicants observed that the number of cfu was reduced by about three orders of magnitude after transformation of the pCRISPR::rpsL plasmid than the control plasmid ($4.8\times10^5/5.3\times10^2$, FIG. **38***a*), suggesting that selection results from CRISPR-induced death of non-edited cells. To measure the rate at which Cas9 cleavage was inactivated, an important parameter of Applicants' method, Applicants transformed cells with either pCRISPR::rpsL or the control plasmid without the W542 editing oligonucleotide (FIG. **38***a*). This background of CRISPR 'escapers', measured as the ratio of pCRISPR::rpsL/pCRISPR::Ø cfu, was 2.5×10^4 ($1.2\times10^2/4.8\times10^5$). Genotyping eight of these escapers revealed that in all cases there was a deletion of the targeting spacer (FIG. **38***b*). This background was higher than the recombineering efficiency of the rpsL mutation, 5.3×10^{-5}, which suggested that to obtain 65% of edited cells, Cas9 cleavage must induce oligonucleotide recombination. To confirm this, Applicants compared the number of kanamycin- and streptomycin-resistant cfu after transformation of pCRISPR::rpsL or pCRISPR::Ø (FIG. **27***d*). As in the case for *S. pneumoniae*, Applicants observed a modest induction of recombination, about 6.7 fold ($2.0\times10^4/3.0\times10^{-5}$). Taken together, these results indicated that the CRISPR system provided a method for selecting mutations introduced by recombineering.

[0281] Applicants showed that CRISPR-Cas systems may be used for targeted genome editing in bacteria by the co-introduction of a targeting construct that killed wild-type cells and an editing template that both eliminated CRISPR cleavage and introduced the desired mutations. Different types of mutations (insertions, deletions or scar-less single-nucleotide substitutions) may be generated. Multiple mutations may be introduced at the same time. The specificity and versatility of editing using the CRISPR system relied on several unique properties of the Cas9 endonuclease: (i) its target specificity may be programmed with a small RNA, without the need for enzyme engineering, (ii) target specificity was very high, determined by a 20 bp RNA-DNA interaction with low probability of non-target recognition, (iii) almost any sequence may be targeted, the only requirement being the presence of an adjacent NGG sequence, (iv) almost any mutation in the NGG sequence, as well as mutations in the seed sequence of the protospacer, eliminates targeting.

[0282] Applicants showed that genome engineering using the CRISPR system worked not only in highly recombinogenic bacteria such as *S. pneumoniae*, but also in *E. coli*.

Results in *E. coli* suggested that the method may be applicable to other microorganisms for which plasmids may be introduced. In *E. coli*, the approach complements recombineering of mutagenic oligonucleotides. To use this methodology in microbes where recombineering is not a possible, the host homologous recombination machinery may be used by providing the editing template on a plasmid. In addition, because accumulated evidence indicates that CRISPR-mediated cleavage of the chromosome leads to cell death in many bacteria and archaea, it is possible to envision the use of endogenous CRISPR-Cas systems for editing purposes.

[0283] In both *S. pneumoniae* and *E. coli*, Applicants observed that although editing was facilitated by a co-selection of transformable cells and a small induction of recombination at the target site by Cas9 cleavage, the mechanism that contributed the most to editing was the selection against non-edited cells. Therefore the major limitation of the method was the presence of a background of cells that escape CRISPR-induced cell death and lack the desired mutation. Applicants showed that these 'escapers' arose primarily through the deletion of the targeting spacer, presumably after the recombination of the repeat sequences that flank the targeting spacer. Future improvements may focus on the engineering of flanking sequences that can still support the biogenesis of functional crRNAs but that are sufficiently different from one another to eliminate recombination. Alternatively, the direct transformation of chimeric crRNAs may be explored. In the particular case of *E. coli*, the construction of the CRISPR-Cas system was not possible if this organism was also used as a cloning host. Applicants solved this issue by placing Cas9 and the tracrRNA on a different plasmid than the CRISPR array. The engineering of an inducible system may also circumvent this limitation.

[0284] Although new DNA synthesis technologies provide the ability to cost-effectively create any sequence with a high throughput, it remains a challenge to integrate synthetic DNA in living cells to create functional genomes. Recently, the co-selection MAGE strategy was shown to improve the mutation efficiency of recombineering by selecting a subpopulation of cells that has an increased probability to achieve recombination at or around a given locus. In this method, the introduction of selectable mutations is used to increase the chances of generating nearby non-selectable mutations. As opposed to the indirect selection provided by this strategy, the use of the CRISPR system makes it possible to directly select for the desired mutation and to recover it with a high efficiency. These technologies add to the toolbox of genetic engineers, and together with DNA synthesis, they may substantially advance both the ability to decipher gene function and to manipulate organisms for biotechnological purposes. Two other studies also relate to CRISPR-assisted engineering of mammalian genomes. It is expected that these crRNA-directed genome editing technologies may be broadly useful in the basic and medical sciences.

[0285] Strains and Culture Conditions.

[0286] *S. pneumoniae* strain R6 was provided by Dr. Alexander Tomasz. Strain crR6 was generated in a previous study. Liquid cultures of *S. pneumoniae* were grown in THYE medium (30 g/l Todd-Hewitt agar, 5 g/l yeast extract). Cells were plated on tryptic soy agar (TSA) supplemented with 5% defibrinated sheep blood. When appropriate, antibiotics were added as followings: kanamycin (400 μg/ml), chloramphenicol (5 μg/ml), erythromycin (1 μg/ml) streptomycin (100 μg/ml) or spectinomycin (100 μg/ml). Measurements of β-galactosidase activity were made using the Miller assay as previously described.

[0287] *E. coli* strains MG1655 and HME63 (derived from MG1655, Δ(argF-lac) U169 λ cI857 Δcro-bioA galK tyr 145 UAG mutS<> amp) (31) were provided by Jeff Roberts and Donald Court, respectively. Liquid cultures of *E. coli* were grown in LB medium (Difco). When appropriate, antibiotics were added as followings: chloramphenicol (25 μg/ml), kanamycin (25 μg/ml) and streptomycin (50 μg/ml).

[0288] *S. pneumoniae* Transformation.

[0289] Competent cells were prepared as described previously (23). For all genome editing transformations, cells were gently thawed on ice and resuspended in 10 volumes of M2 medium supplemented with 100 ng/ml of competence-stimulating peptide CSP1(40), and followed by addition of editing constructs (editing constructs were added to cells at a final concentration between 0.7 ng/gl to 2.5 μg/ul). Cells were incubated 20 min at 37° C. before the addition of 2 μl of targeting constructs and then incubated 40 min at 37° C. Serial dilutions of cells were plated on the appropriate medium to determine the colony forming units (cfu) count.

[0290] *E. coli* Lambda-Red Recombineering. Strain HME63 was used for all recombineering experiments. Recombineering cells were prepared and handled according to a previously published protocol (6). Briefly, a 2 ml overnight culture (LB medium) inoculated from a single colony obtained from a plate was grown at 30° C. The overnight culture was diluted 100-fold and grown at 30° C. with shaking (200 rpm) until the OD_{600} is from 0.4-0.5 (approximately 3 hrs). For Lambda-red induction, the culture was transferred to a 42° C. water bath to shake at 200 rpm for 15 min. Immediately after induction, the culture was swirled in an ice-water slurry and chilled on ice for 5-10 min. Cells were then washed and aliquoted according to the protocol. For electro-transformation, 50 μl of cells were mixed with 1 mM of salt-free oligos (IDT) or 100-150 ng of plasmid DNA (prepared by QIAprep Spin Miniprep Kit, Qiagen). Cells were electroporated using 1 mm Gene Pulser cuvette (Bio-rad) at 1.8 kV and were immediately resuspended in 1 ml of room temperature LB medium. Cells were recovered at 30° C. for 1-2 hrs before being plated on LB agar with appropriate antibiotic resistance and incubated at 32° C. overnight.

[0291] Preparation of *S. pneumoniae* Genomic DNA.

[0292] For transformation purposes, *S. pneumoniae* genomic DNA was extracted using the Wizard Genomic DNA Purification Kit, following instructions provided by the manufacturer (Promega). For genotyping purposes, 700 ul of overnight *S. pneumoniae* cultures were pelleted, resuspended in 60 ul of lysozyme solution (2 mg/ml) and incubated 30 min at 37° C. The genomic DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen).

[0293] Strain Construction.

[0294] All primers used in this study are provided in Table G. To generate *S. pneumoniae* crR6M, an intermediate strain, LAM226, was made. In this strain the aphA-3 gene (providing kanamycin resistance) adjacent to the CRISPR array of *S. pneumoniae* crR6 strain was replaced by a cat gene (providing chloramphenicol resistance). Briefly, crR6 genomic DNA was amplified using primers L448/L444 and L447/L481, respectively. The cat gene was amplified from plasmid pC194 using primers L445/L446. Each PCR product was gel-purified and all three were fused by SOEing PCR with primers L448/L481. The resulting PCR product was transformed into

competent *S. pneumoniae* crR6 cells and chloramphenicol-resistant transformants were selected. To generate *S. pneumoniae* crR6M, *S. pneumoniae* crR6 genomic DNA was amplified by PCR using primers L409/L488 and L448/L481, respectively. Each PCR product was gel-purified and they were fused by SOEing PCR with primers L409/L481. The resulting PCR product was transformed into competent *S. pneumoniae* LAM226 cells and kanamycin-resistant transformants were selected.

[0295] To generate *S. pneumoniae* crR6Rc, *S. pneumoniae* crR6M genomic DNA was amplified by PCR using primers L430/W286, and *S. pneumoniae* LAM226 genomic DNA was amplified by PCR using primers W288/L481. Each PCR product was gel-purified and they were fused by SOEing PCR with primers L430/L481. The resulting PCR product was transformed into competent *S. pneumoniae* crR6M cells and chloramphenicol-resistant transformants were selected.

[0296] To generate *S. pneumoniae* crR6Rk, *S. pneumoniae* crR6M genomic DNA was amplified by PCR using primers L430/W286 and W287/L481, respectively. Each PCR product was gel-purified and they were fused by SOEing PCR with primers L430/L481. The resulting PCR product was transformed into competent *S. pneumoniae* crR6Rc cells and kanamycin-resistant transformants were selected.

[0297] To generate JEN37, *S. pneumoniae* crR6Rk genomic DNA was amplified by PCR using primers L430/W356 and W357/L481, respectively. Each PCR product was gel-purified and they were fused by SOEing PCR with primers L430/L481. The resulting PCR product was transformed into competent *S. pneumoniae* crR6Rc cells and kanamycin-resistant transformants were selected.

[0298] To generate JEN38, R6 genomic DNA was amplified using primers L422/L461 and L459/L426, respectively. The ermAM gene (specifying erythromycin resistance) was amplified from plasmid pFW15[43] using primers L457/L458. Each PCR product was gel-purified and all three were fused by SOEing PCR with primers L422/L426. The resulting PCR product was transformed into competent *S. pneumoniae* crR6Rc cells and erythromycin-resistant transformants were selected.

[0299] *S. pneumoniae* JEN53 was generated in two steps. First JEN43 was constructed as illustrated in FIG. **33**. JEN53 was generated by transforming genomic DNA of JEN25 into competent JEN43 cells and selecting on both chloramphenicol and erythromycin.

[0300] To generate *S. pneumoniae* JEN62, *S. pneumoniae* crR6Rk genomic DNA was amplified by PCR using primers W256/W365 and W366/L403, respectively. Each PCR product was purified and ligated by Gibson assembly. The assembly product was transformed into competent *S. pneumoniae* crR6Rc cells and kanamycin-resistant transformants were selected.

[0301] Plasmid construction. pDB97 was constructed through phosphorylation and annealing of oligonucleotides B296/B297, followed by ligation in pLZ12spec digested by EcoRI/BamHI. Applicants fully sequenced pLZ12spec and deposited its sequence in genebank (accession: KC112384).

[0302] pDB98 was obtained after cloning the CRISPR leader sequence was cloned together with a repeat-spacer-repeat unit into pLZ12spec. This was achieved through amplification of crR6Rc DNA with primers B298/B320 and B299/B321, followed by SOEing PCR of both products and cloning in pLZ12spec with restriction sites BamHI/EcoRI. In this way the spacer sequence in pDB98 was engineered to contain two BsaI restriction sites in opposite directions that allow for the scar-less cloning of new spacers.

[0303] pDB99 to pDB108 were constructed by annealing of oligonucleotides B300/B301 (pDB99), B302/B303 (pDB100), B304/B305 (pDB101), B306/B307 (pDB102), B308/B309 (pDB103), B310/B311 (pDB104), B312/B313 (pDB105), B314/B315 (pDB106), B315/B317 (pDB 107), B318/B319 (pDB 108), followed by ligation in pDB98 cut by BsaI.

[0304] The pCas9 plasmid was constructed as follow. Essential CRISPR elements were amplified from *Streptococcos pyogenes* SF370 genomic DNA with flanking homology arms for Gibson Assembly. The tracrRNA and Cas9 were amplified with oligos HC008 and HC010. The leader and CRISPR sequences were amplified HC011/HC014 and HC015/HC009, so that two BsaI type IIS sites were introduced in between two direct repeats to facilitate easy insertion of spacers.

[0305] pCRISPR was constructed by subcloning the pCas9 CRISPR array in pZE21-MCS1 through amplification with oligos B298+B299 and restriction with EcoRI and BamHI. The rpsL targeting spacer was cloned by annealing of oligos B352+B353 and cloning in the BsaI cut pCRISPR giving pCRISPR::rpsL.

[0306] Generation of Targeting and Editing Constructs.

[0307] Targeting constructs used for genome editing were made by Gibson assembly of Left PCRs and Right PCRs (Table G). Editing constructs were made by SOEing PCR fusing PCR products A (PCR A), PCR products B (PCR B) and PCR products C(PCR C) when applicable (Table G). The CRISPR::Ø and CRISPR::ermAM(stop) targeting constructs were generated by PCR amplification of JEN62 and crR6 genomic DNA respectively, with oligos L409 and L481.

[0308] Generation of Targets with Randomized PAM or Protospacer Sequences.

[0309] The 5 nucleotides following the spacer 1 target were randomized through amplification of $R6^{8232.5}$ genomic DNA with primers W377/L426. This PCR product was then assembled with the cat gene and the srtA upstream region that were amplified from the same template with primers L422/W376. 80 ng of the assembled DNA was used to transform strains R6 and crR6. Samples for the randomized targets were prepared using the following primers: B280-B290/L426 to randomize bases 1-10 of the target and B269-B278/L426 to randomize bases 10-20. Primers L422/B268 and L422/B279 were used to amplify the cat gene and srtA upstream region to be assembled with the first and last 10 PCR products respectively. The assembled constructs were pooled together and 30 ng was transformed in R6 and crR6. After transformation, cells were plated on chloramphenicol selection. For each sample more than 2×10^5 cells were pooled together in 1 ml of THYE and genomic DNA was extracted with the Promega Wizard kit. Primers B250/B251 were used to amplify the target region. PCR products were tagged and run on one Illumina MiSeq paired-end lane using 300 cycles.

[0310] Analysis of Deep Sequencing Data.

[0311] Randomized PAM: For the randomized PAM experiment 3,429,406 reads were obtained for crR6 and 3,253,998 for R6. It is expected that only half of them will correspond to the PAM-target while the other half will sequence the other end of the PCR product. 1,623,008 of the crR6 reads and 1,537,131 of the R6 reads carried an error-free target sequence. The occurrence of each possible PAM among these reads is shown in supplementary file. To estimate the functionality of a PAM, its relative proportion in the

crR6 sample over the R6 sample was computed and is denoted r_{ijklm} where I,j,k,l,m are one of the 4 possible bases. The following statistical model was constructed:

$$\log(r_{ijklm})=\mu+b2_i+b3_j+b4_k+b2b3_{i,j}+b3b4_{j,k}+\epsilon_{ijklm},$$

[0312] where ϵ is the residual error, b2 is the effect of the 2^{nd} base of the PAM, b3 of the third, b4 of the fourth, b2b3 is the interaction between the second and third bases, b3b4 between the third and fourth bases. An analysis of variance was performed:

Anova table

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
b3	3	151.693	50.564	601.8450	<2.2e−16 ***
b2	3	90.521	30.174	359.1454	<2.2e−16 ***
b4	3	1.881	0.627	7.4623	6.070e−05 ***
b3:b2	9	228.940	25.438	302.7738	<2.2e−16 ***
b3:b4	9	3.010	0.334	3.9809	5.227e−05 ***
Residuals	996	83.680	0.084		

[0313] When added to this model, b1 or b5 do not appear to be significant and other interactions than the ones included can also be discarded. The model choice was made through successive comparisons of more or less complete models using the anova method in R. Tukey's honest significance test was used to determine if pairwise differences between effects are significant.

[0314] NGGNN patterns are significantly different from all other patterns and carry the strongest effect (see table below).

[0315] In order to show that positions 1, 4 or 5 do not affect the NGGNN pattern Applicants looked at theses sequences only. Their effect appears to be normally distributed (see QQ plot in FIG. **71**), and model comparisons using the anova method in R shows that the null model is the best one, i.e. there is no significant role of b1, b4 and b5.

[0316] Model Comparison Using the Anova Method in R for the NGGNN Sequences

Model 1:	ratio.log ~ 1					
Model 2:	ratio.log ~ b1 + b4 + b5					
	Res. Df	RSS	Df	Sum of Sq	F	Pr(>F)
1	63	14.579				
2	54	11.295	9	3.2836	1.7443	0.1013

[0317] Partial Interference of NAGNN and NNGGN Patterns

[0318] NAGNN patterns are significantly different from all other patterns but carry a much smaller effect than NGGNN (see Tukey's honest significance test below).

[0319] Finally, NTGGN and NCGGN patterns are similar and show significantly more CRISPR interference than NTGHN and NCGHN patterns (where H is A,T or C), as shown by a bonferroni adjusted pairwise student-test.

[0320] Pairwise Comparisons of the Effect of b4 on NYGNN Sequences Using t Tests with Pooled SD

Data: b4

	A	C	G
C	1.00	—	—
G	9.2e−05	2.4e−06	—
T	0.31	1.00	1.2e−08

[0321] Taken together, these results allow concluding that NNGGN patterns in general produce either a complete interference in the case of NGGGN, or a partial interference in the case of NAGGN, NTGGN or NCGGN.

[0322] Tukey multiple comparisons of means: 95% family-wise confidence level

	diff	lwr	upr	p adj
		$b2:b3		
G:G-A:A	−2.76475	−2.94075	−2.58875	<1E−07
G:G-C:A	−2.79911	−2.97511	−2.62311	<1E−07
G:G-T:A	−2.7809	−2.9569	−2.6049	<1E−07
G:G-A:C	−2.81643	−2.99244	−2.64043	<1E−07
G:G-C:C	−2.77903	−2.95504	−2.60303	<1E−07
G:G-G:C	−2.64867	−2.82468	−2.47267	<1E−07
G:G-T:C	−2.79718	−2.97319	−2.62118	<1E−07
G:G-A:G	−2.67068	−2.84668	−2.49468	<1E−07
G:G-C:G	−2.73525	−2.91125	−2.55925	<1E−07
G:G-T:G	−2.7976	−2.62159	−2.9736	<1E−07
G:G-A:T	−2.76727	−2.59127	−2.94328	<1E−07
G:G-C:T	−2.84114	−2.66513	−3.01714	<1E−07
G:G-G:T	−2.76409	−2.58809	−2.94009	<1E−07
G:G-T:T	−2.76781	−2.59181	−2.94381	<1E−07
G:G-G:A	−2.13964	−2.31565	−1.96364	<1E−07
G:A-A:A	−0.62511	−0.80111	−0.4491	<1E−07
G:A-C:A	−0.65947	−0.83547	−0.48346	<1E−07
G:A-T:A	−0.64126	−0.46525	−0.81726	<1E−07
G:A-A:C	−0.67679	−0.50078	−0.85279	<1E−07
G:A-C:C	−0.63939	−0.46339	−0.81539	<1E−07
G:A-G:C	−0.50903	−0.33303	−0.68503	<1E−07
G:A-T:C	−0.65754	−0.48154	−0.83354	<1E−07
G:A-A:G	−0.53104	−0.35503	−0.70704	<1E−07
G:A-C:G	−0.59561	−0.4196	−0.77161	<1E−07
G:A-T:G	−0.65795	−0.48195	−0.83396	<1E−07
G:A-A:T	−0.62763	−0.45163	−0.80363	<1E−07
G:A-C:T	−0.70149	−0.52549	−0.8775	<1E−07
G:A-G:T	−0.62445	−0.44844	−0.80045	<1E−07
G:A-T:T	−0.62817	−0.45216	−0.80417	<1E−07
		$b3:b4		
G:G-G:A	−0.33532	−0.51133	−0.15932	<1E−07
G:G-G:C	−0.18113	−0.35719	−0.00518	0.036087
G:G-G:T	−0.31626	−0.14026	−0.49226	<1E−07

[0323] Randomized Target

[0324] For the randomized target experiment 540,726 reads were obtained for crR6 and 753,570 for R6. As before, only half of the reads are expected to sequence the interesting end of the PCR product. After filtering for reads that carry a target that is error-free or with a single point mutation, 217,656 and 353,141 reads remained for crR6 and R6 respectively. The relative proportion of each mutant in the crR6 sample over the R6 sample was computed (FIG. **24***c*). All mutations outside of the seed sequence (13-20 bases away from the PAM) show full interference. Those sequences were used as a reference to determine if other mutations inside the seed sequence can be said to significantly disrupt interference. A normal distribution was fitted to theses sequences using the fitdistr function of the MASS R package. The 0.99 quantile of the fitted distribution is shown as a dotted line in FIG. **24***c*. FIG. **72** shows a histogram of the data density with fitted normal distribution (black line) and 0.99 quantile (dotted line).

TABLE F

Relative abundance of PAM sequences in the crR6/R6 samples averaged over bases 1 and 5.

2nd position	3rd position A	3rd position C	3rd position G	3rd position T	4th position
A	**AAA** 1.04	**ACA** 1.12	AGA 0.73	**ATA** 1.10	A
	AAC 1.07	**ACC** 1.04	AGC 0.64	**ATC** 0.97	C
	AAG 1.00	**ACG** 1.09	AGG 0.61	**ATG** 1.07	G
	AAT 0.98	**ACT** 1.02	AGT 0.65	**ATT** 1.01	T
C	**CAA** 1.05	**CCA** 1.05	**CGA** 0.99	**CTA** 1.07	A
	CAC 1.04	**CCC** 1.02	**CGC** 1.08	**CTC** 1.04	C
	CAG 1.08	**CCG** 1.08	CGG 0.81	**CTG** 1.05	G
	CAT 1.13	**CCT** 1.05	**CGT** 1.07	**CTT** 1.08	T

TABLE F-continued

Relative abundance of PAM sequences in the crR6/R6 samples averaged over bases 1 and 5.

2nd position	3rd position A	3rd position C	3rd position G	3rd position T	4th position
G	**GAA** 0.97	**GCA** 1.05	**GGA** 0.08	**GTA** 0.99	A
	GAC 0.92	**GCC** 1.00	**GGC** 0.05	**GTC** 1.15	C
	GAG 0.96	**GCG** 0.98	**GGG** 0.07	**GTG** 0.98	G
	GAT 0.98	**GCT** 0.99	**GGT** 0.06	**GTT** 1.05	T
T	**TAA** 1.08	**TCA** 1.16	**TGA** 1.05	**TTA** 1.14	A
	TAC 1.00	**TCC** 1.08	**TGC** 1.08	**TTC** 1.05	C
	TAG 1.02	**TCG** 1.11	TGG 0.77	**TTG** 1.01	G
	TAT 1.01	**TCT** 1.12	**TGT** 1.21	**TTT** 1.02	T

TABLE G

Primers used in this study (SEQ ID NOS 68-183, respectively, in order of appearance).

Primer	Sequence 5'-3'
B217	TCCTAGCAGGATTTCTGATATTACTGTCACGTTTTAGAGCTATGCTGTTTTGA
B218	GTGACAGTAATATCAGAAATCCTGCTAGGAGTTTTGGGACCATTCAAAACAGC
B229	GGGTTTCAAGTCTTTGTAGCAAGAG
B230	GCCAATGAACGGGAACCCTTGGTC
B250	NNNNGACGAGCAATGGCTGAAATC
B251	NNNNTTATTTGGCTCATATTTGCTG
B255	CTTTACACCAATCGCTGCAACAGAC
B256	CAAAATTTCTAGTCTTCTTTGCCTTTCCCCATAAAACCCTCCTTA
B257	AGGGTTTTATGGGGAAAGGCAAAGAAGACTAGAAATTTTGATACC
B258	CTTACGGTGCATAAAGTCAATTTCC
B269	TGGCTCGATTTCAGCCATTGC
B270	CTTTGACGAGGCAATGGCTGAAATCGAGCCAANAAAGCGCAAG
B271	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAANAAGCGCAAG
B272	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAANAGCGCAAG
B273	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAANGCGCAAG
B274	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAANCGCAAG
B275	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAAGNGCAAG
B276	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAAGCNCAAGAAG
B277	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAAGCGNAAGAAG
B278	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAAGCGCNAGAAG
B279	GCGCTTTTTTGGCTCGATTTCAG
B280	CAATGGCTGAAATCGAGCCAAAAAAGCGCANGAAGAAATC
B281	CAATGGCTGAAATCGAGCCAAAAAAGCGCAANAAGAAATC
B282	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGNAGAAATC
B283	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGANGAAATC

TABLE G-continued

Primers used in this study (SEQ ID NOS 68-183, respectively, in order of appearance).

Primer	Sequence 5'-3'
B284	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAANAAATC
B285	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGNAATCAACC
B286	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGANATCAACC
B287	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGAANTCAACC
B288	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGAAANCAACC
B289	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGAAATNAACCAGC
B290	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGAAATCNACCAGC
B296	gatccTCCATCCGTACAACCCACAACCCTGg
B297	aattcCAGGGTTGTGGGTTGTACGGATGGAg
B298	CATGGATCCTATTTCTTAATAACTAAAAATATGG
B299	CATGAATTCAACTCAACAAGTCTCAGTGTGCTG
B300	AAACATTTTTTCTCCATTTAGGAAAAAGGATGCTG
B301	AAAACAGCATCCTTTTTCCTAAATGGAGAAAAAAT
B302	AAACCTTAAATCAGTCACAAATAGCAGCAAAATTG
B303	AAAACAATTTTGCTGCTATTTGTGACTGATTTAAG
B304	AAACTTTTCATCATACGACCAATCTGCTTTATTTG
B305	AAAACAAATAAAGCAGATTGGTCGTATGATGAAAA
B306	AAACTCGTCCAGAAGTTATCGTAAAAGAAATCGAG
B307	AAAACTCGATTTCTTTTACGATAACTTCTGGACGA
B308	AAACAATCTCTCCAAGGTTTCCTTAAAAATCTCTG
B309	AAAACAGAGATTTTTAAGGAAACCTTGGAGAGATT
B310	AAACGCCATCGTCAGGAAGAAGCTATGCTTGAGTG
B311	AAAACACTCAAGCATAGCTTCTTCCTGACGATGGC
B312	AAACATCTCTATACTTATTGAAATTTCTTTGTATG
B313	AAAACATACAAAGAAATTTCAATAAGTATAGAGAT
B314	AAACTAGCTGTGATAGTCCGCAAAACCAGCCTTCG
B315	AAAACGAAGGCTGGTTTTGCGGACTATCACAGCTA
B316	AAACATCGGAAGGTCGAGCAAGTAATTATCTTTTG
B317	AAAACAAAAGATAATTACTTGCTCGACCTTCCGAT
B318	AAACAAGATGGTATCGCAAAGTAAGTGACAATAAG
B319	AAAACTTATTGTCACTTACTTTGCGATACCATCTT
B320	GAGACCTTTGAGCTTCCGAGACTGGTCTCAGTTTTGGGACCATTCAAAACAG
B321	TGAGACCAGTCTCGGAAGCTCAAAGGTCTCGTTTTAGAGCTATGCTGTTTTG
B352	aaacTACTTTACGCAGCGCGGAGTTCGGTTTTTTg
B353	aaaacAAAAAACCGAACTCCGCGCTGCGTAAAGTA
HC008_SP	ATGCCGGTACTGCCGGGCCTCTTGCGGGATTACGAAATCATCCTG

TABLE G-continued

Primers used in this study (SEQ ID NOS 68-183, respectively, in order of appearance).

Primer	Sequence 5'-3'
HC009_SP	GTGACTGGCGATGCTGTCGGAATGGACGATCACACTACTCTTCTT
HC010_SP	TTAAGAAATAATCTTCATCTAAAATATACTTCAGTCACCTCCTAGCTGAC
HC011_SP	ATTGATTTGAGTCAGCTAGGAGGTGACTGAAGTATATTTTAGATGAAG
HC014_SP	GAGACCTTTGAGCTTCCGAGACTGGTCTCAGTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCTCGTAGACTA TTTTTGTC
HC015_SP	GAGACCAGTCTCGGAAGCTCAAAGGTCTCGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACTTCAGCACACTG AGACTTG
L403	AGTCATCCCAGCAACAAATGG
L409	CGTGGTAAATCGGATAACGTTCCAAGTGAAG
L422	Tgctcttcttcacaaacaaggg
L426	AAGCCAAAGTTTGGCACCACC
L430	GTAGCTTATTCAGTCCTAGTGG
L444	CGTTTGTTGAACTAATGGGTGCAAATTACGAATCTTCTCCTGACG
L445	CGTCAGGAGAAGATTCGTAATTTGCACCCATTAGTTCAACAAACG
L446	GATATTATGGAGCCTATTTTTGTGGGTTTTTAGGCATAAAACTATATG
1447	CATATAGTTTTATGCCTAAAAACCcACAAAAATAGGCTCCATAATATC
L448	ATTATTTCTTAATAACTAAAAATATGG
L457	CGTgtacaattgctagcgtacggc
L458	GCACCGGTGATCACTAGTCCTAGG
L459	cctaggactagtgatcaccggtGCAAATATGAGCCAAATAAATATAT
L461	GCCGTACGCTAGCAATTGTACACGTTTGTTGAACTAATGGGTGC
L481	TTCAAATTTTCCCATTTGATTCTCC
L488	CCATATTTTTAGTTATTAAGAAATAATACCAGCCATCAGTCACCTCC
W256	AGACGATTCAATAGACAATAAGG
W286	GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCTCGTAGAC
W287	GCTATGCTGTTTTGAATGGTCCCAAAACcattattttaacacacgaggtg
W288	GCTATGCTGTTTTGAATGGTCCCAAAACGCACCCATTAGTTCAACAAACG
W326	AATTCTTTTCTTCATCATCGGTC
W327	AAGAAAGAATGAAGATTGTTCATG
W341	GGTACTAATCAAAATAGTGAGGAGG
W354	GTTTTTCAAAATCTGCGGTTGCG
W355	AAAAATTGAAAAAATGGTGGAAACAC
W356	ATTTCGTAAACGGTATCGGTTTCTTTTAAAGTTTTGGGACCATTCAAAACAGC
W357	TTTAAAAGAAACCGATACCGTTTACGAAATGTTTTAGAGCTATGCTGTTTTGA
W365	AAACGGTATCGGTTTCTTTTAAATTCAATTGTTTTGGGACCATTCAAAACAGC
W366	AATTGAATTTAAAAGAAACCGATACCGTTTGTTTTAGAGCTATGCTGTTTTGA
W370	GTTCCTTAAACCAAAACGGTATCGGTTTCTTTTAAATTC

TABLE G-continued

Primers used in this study (SEQ ID NOS 68-183, respectively, in order of appearance).

Primer	Sequence 5'-3'
W371	GAAACCGATACCGTTTTGGTTTAAGGAACAGGTAAAGGGCATTTAAC
W376	CGATTTCAGCCATTGCCTCGTC
W377	GCCTTTGACGAGGCAATGGCTGAAATCGNNNNNAAAAAGCGCAAGAAGAAATCAAC
W391	TCCGTACAACCCACAACCCTGCTAGTGAGCGTTTTGGGACCATTCAAAACAGC
W392	GCTCACTAGCAGGGTTGTGGGTTGTACGGAGTTTTAGAGCTATGCTGTTTTGA
W393	TTGTTGCCACTCTTCCTTCTTTC
W397	CAGGGTTGTGGGTTGTTGCGATGGAGTTAACTCCCATCTCC
W398	GGGAGTTAACTCCATCGCAACAACCCACAACCCTGCTAGTG
W403	GTGGTATCTATCGTGATGTGAC
W404	TTACCGAAACGGAATTTATCTGC
W405	AAAGCTAGAGTTCCGCAATTGG
W431	GTGGGTTGTACGGATTGAGTTAACTCCCATCTCCTTC
W432	GATGGGAGTTAACTCAATCCGTACAACCCACAACCCTG
W433	GCTTCACCTATTGCAGCACCAATTGACCACATGAAGATAG
W434	GTGGTCAATTGGTGCTGCAATAGGTGAAGCTAATGGTGATG
W463	CTGATTTGTATTAATTTTGAGACATTATGCTTCACCTTC
W464	GCATAATGTCTCAAAATTAATACAAATCAGTGAAATCATG
W465	GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACGTGACAGTAATATCAG
W466	GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGCTCACTAGCAGGGTTG
W542	ATACTTTACGCAGCGCGGAGTTCGGTTTTgTAGGAGTGGTAGTATATACACGAGTACAT

TABLE H

Design of targeting and editing constructs used in this study (SEQ ID NOS 184, 184, 184, 185, and 186, respectively, in order of appearance).

	Targeting Controls					Editing Constructs
	Template					
Edition	DNA	Left PCR	Right PCR	Spacer sequence	PAM	Template DNA
? R > A	?k	W256W39?	W3?403	GCTCACT AGC AGGGTTGTGGGTTGT ACGGA	TGG	R6
bgaA NE > AA	?k	W256W39	W392?403	GCTCACT AGC AGGGTTGTGGGTTGT ACGGA	TGG	R6
?A	?k	W256W39	W392?403	GCTCACT AGC AGGGTTGTGGGTTGT ACGGA	TGG	R6
?A	?c	W25621?	?2?413	TCCTAGCAGGATTTCTGATATTACTGTCAC	TGG	R6
?B ?	?k	W256W356	W357?403	TTT AAAAGAAACCGAT ACCGTTTACGAAAT	TGG	JEN38
? ?	EN51 (for Left PCR) and EN52 (for Right PCR)	W256W465	W416W403	?	TGG	?

TABLE H-continued

Design of targeting and editing constructs used in this study (SEQ ID NOS 184, 184, 184, 185, and 186, respectively, in order of appearance).

	Editing Constructs				Name of resulting	Primers used to verify edited
Edition	PCR A	PCR B	PCR C	⍰ Flag PCR	strains	genotype
⍰ R > A	W403/W397	W3⍰/W414	N/A	W⍰3/W404	JEN56	W403/W404
bgaA NE > AA	W403/W453	W432/W433	W434/W404	W⍰3/W404	JEN60	W403/W404
⍰A	B255/⍰256	B2⍰/B258	N/A	B255/B25⍰	JEN⍰1	W403/W405
⍰A	B230/W⍰	W464/W429	N/A	B230/⍰	JEN⍰1	W472/W431
⍰B ⍰	L422/W320	W371/W426	N/A	L422/L421	JEN43	L457/L458
⍰ ⍰					JEN64	⍰ ⍰ ⍰

⍰ indicates text missing or illegible when filed

Example 6

Optimization of the Guide RNA for *Streptococcus pyogenes* Cas9 (Referred to as SpCas9)

[0325] Applicants mutated the tracrRNA and direct repeat sequences, or mutated the chimeric guide RNA to enhance the RNAs in cells.

[0326] The optimization is based on the observation that there were stretches of thymines (Ts) in the tracrRNA and guide RNA, which might lead to early transcription termination by the pol 3 promoter. Therefore Applicants generated the following optimized sequences. Optimized tracrRNA and corresponding optimized direct repeat are presented in pairs.

Optimized tracrRNA 1 (mutation underlined):
(SEQ ID NO: 187)
GGAACCATTCAtAACAGCATAGCAAGTTAtAATAAGGCTAGTCCGTTAT
CAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTT

Optimized direct repeat 1 (mutation underlined):
(SEQ ID NO: 188)
GTTaTAGAGCTATGCTGTTaTGAATGGTCCCAAAAC

Optimized tracrRNA 2 (mutation underlined):
(SEQ ID NO: 189)
GGAACCATTCAAtACAGCATAGCAAGTTAAtATAAGGCTAGTCCGTTAT
CAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTT

Optimized direct repeat 2 (mutation underlined):
(SEQ ID NO: 190)
GTaTTAGAGCTATGCTGTaTTGAATGGTCCCAAAAC

[0327] Applicants also optimized the chimeric guideRNA for optimal activity in eukaryotic cells.

Original guide RNA:
(SEQ ID NO: 191)
NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAA
TAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
TTTTT

Optimized chimeric guide RNA sequence 1:
(SEQ ID NO: 192)
NNNNNNNNNNNNNNNNNNNNGTATTAGAGCTAGAAATAGCAAGTTAATA
TAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
TTTTT

-continued

Optimized chimeric guide RNA sequence 2:
(SEQ ID NO: 193)
NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTATGCTGTTTTGGAAACAA
AACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAA
GTGGCACCGAGTCGGTGCTTTTTTT

Optimized chimeric guide RNA sequence 3:
(SEQ ID NO: 194)
NNNNNNNNNNNNNNNNNNNNGTATTAGAGCTATGCTGTATTGGAAACAA
TACAGCATAGCAAGTTAATATAAGGCTAGTCCGTTATCAACTTGAAAAA
GTGGCACCGAGTCGGTGCTTTTTTT

[0328] Applicants showed that optimized chimeric guide RNA works better as indicated in FIG. **3**. The experiment was conducted by co-transfecting 293FT cells with Cas9 and a U6-guide RNA DNA cassette to express one of the four RNA forms shown above. The target of the guide RNA is the same target site in the human Emxl locus: "GTCACCTCCAATGACTAGGG (SEQ ID NO: 195)"

Example 7

Optimization of *Streptococcus thermophiles* LMD-9 CRISPR1 Cas9 (Referred to as St1Cas9)

[0329] Applicants designed guide chimeric RNAs as shown in FIG. **4**.

[0330] The StlCas9 guide RNAs can undergo the same type of optimization as for SpCas9 guide RNAs, by breaking the stretches of poly thymines (Ts)

Example 8

Cas9 Diversity and Mutations

[0331] The CRISPR-Cas system is an adaptive immune mechanism against invading exogenous DNA employed by diverse species across bacteria and archaea. The type II CRISPR-Cas9 system consists of a set of genes encoding proteins responsible for the "acquisition" of foreign DNA into the CRISPR locus, as well as a set of genes encoding the "execution" of the DNA cleavage mechanism; these include the DNA nuclease (Cas9), a non-coding transactivating crRNA (tracrRNA), and an array of foreign DNA-derived spacers flanked by direct repeats (crRNAs). Upon maturation by Cas9, the tracRNA and crRNA duplex guide the Cas9 nuclease to a target DNA sequence specified by the spacer guide sequences, and mediates double-stranded breaks in the

DNA near a short sequence motif in the target DNA that is required for cleavage and specific to each CRISPR-Cas system. The type II CRISPR-Cas systems are found throughout the bacterial kingdom and highly diverse in in Cas9 protein sequence and size, tracrRNA and crRNA direct repeat sequence, genome organization of these elements, and the motif requirement for target cleavage. One species may have multiple distinct CRISPR-Cas systems.

[0332] Applicants evaluated 207 putative Cas9s from bacterial species identified based on sequence homology to known Cas9s and structures orthologous to known subdomains, including the HNH endonuclease domain and the RuvC endonuclease domains [information from the Eugene Koonin and Kira Makarova]. Phylogenetic analysis based on the protein sequence conservation of this set revealed five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids) (FIGS. **39** and **40**A-F).

[0333] In this example, Applicants show that the following mutations can convert SpCas9 into a nicking enzyme: D10A, E762A, H840A, N854A, N863A, D986A.

[0334] Applicants provide sequences showing where the mutation points are located within the SpCas9 gene (FIG. **41**). Applicants also show that the nickases are still able to mediate homologous recombination (Assay indicated in FIG. **2**). Furthermore, Applicants show that SpCas9 with these mutations (individually) do not induce double strand break (FIG. **47**).

Example 9

Supplement to DNA Targeting Specificity of the RNA-Guided Cas9 Nuclease

[0335] Cell Culture and Transfection

[0336] Human embryonic kidney (HEK) cell line 293FT (Life Technologies) was maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone), 2 mM GlutaMAX (Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° C. with 5% CO2 incubation.

[0337] 293FT cells were seeded either onto 6-well plates, 24-well plates, or 96-well plates (Corning) 24 hours prior to transfection. Cells were transfected using Lipofectamine 2000 (Life Technologies) at 80-90% confluence following the manufacturer's recommended protocol. For each well of a 6-well plate, a total of 1 ug of Cas9+sgRNA plasmid was used. For each well of a 24-well plate, a total of 500 ng Cas9+sgRNA plasmid was used unless otherwise indicated. For each well of a 96-well plate, 65 ng of Cas9 plasmid was used at a 1:1 molar ratio to the U6-sgRNA PCR product.

[0338] Human embryonic stem cell line HUES9 (Harvard Stem Cell Institute core) was maintained in feeder-free conditions on GelTrex (Life Technologies) in mTesR medium (Stemcell Technologies) supplemented with 100 ug/ml Normocin (InvivoGen). HUES9 cells were transfected with Amaxa P3 Primary Cell 4-D Nucleofector Kit (Lonza) following the manufacturer's protocol.

[0339] SURVEYOR Nuclease Assay for Genome Modification

[0340] 293FT cells were transfected with plasmid DNA as described above. Cells were incubated at 37° C. for 72 hours post-transfection prior to genomic DNA extraction. Genomic DNA was extracted using the QuickExtract DNA Extraction Solution (Epicentre) following the manufacturer's protocol. Briefly, pelleted cells were resuspended in QuickExtract solution and incubated at 65° C. for 15 minutes and 98° C. for 10 minutes.

[0341] The genomic region flanking the CRISPR target site for each gene was PCR amplified (primers listed in Tables J and K), and products were purified using QiaQuick Spin Column (Qiagen) following the manufacturer's protocol. 400 ng total of the purified PCR products were mixed with 2 μl 10× Taq DNA Polymerase PCR buffer (Enzymatics) and ultrapure water to a final volume of 20 μl, and subjected to a re-annealing process to enable heteroduplex formation: 95° C. for 10 min, 95° C. to 85° C. ramping at −2° C./s, 85° C. to 25° C. at −0.25° C./s, and 25° C. hold for 1 minute. After re-annealing, products were treated with SURVEYOR nuclease and SURVEYOR enhancer S (Transgenomics) following the manufacturer's recommended protocol, and analyzed on 4-20% Novex TBE poly-acrylamide gels (Life Technologies). Gels were stained with SYBR Gold DNA stain (Life Technologies) for 30 minutes and imaged with a Gel Doc gel imaging system (Bio-rad). Quantification was based on relative band intensities.

[0342] Northern Blot Analysis of tracrRNA Expression in Human Cells

[0343] Northern blots were performed as previously described. Briefly, RNAs were heated to 95° C. for 5 min before loading on 8% denaturing polyacrylamide gels (SequaGel, National Diagnostics). Afterwards, RNA was transferred to a pre-hybridized Hybond N+membrane (GE Healthcare) and crosslinked with Stratagene UV Crosslinker (Stratagene). Probes were labeled with [gamma-32P] ATP (Perkin Elmer) with T4 polynucleotide kinase (New England Biolabs). After washing, membrane was exposed to phosphor screen for one hour and scanned with phosphorimager (Typhoon).

[0344] Bisulfite Sequencing to Assess DNA Methylation Status

[0345] HEK 293FT cells were transfected with Cas9 as described above. Genomic DNA was isolated with the DNeasy Blood & Tissue Kit (Qiagen) and bisulfite converted with EZ DNA Methylation-Lightning Kit (Zymo Research). Bisulfite PCR was conducted using KAPA2G Robust HotStart DNA Polymerase (KAPA Biosystems) with primers designed using the Bisulfite Primer Seeker (Zymo Research, Tables J and K). Resulting PCR amplicons were gel-purified, digested with EcoRI and HindIII, and ligated into a pUC19 backbone prior to transformation. Individual clones were then Sanger sequenced to assess DNA methylation status.

[0346] In Vitro Transcription and Cleavage Assay

[0347] HEK 293FT cells were transfected with Cas9 as described above. Whole cell lysates were then prepared with a lysis buffer (20 mM HEPES, 100 mM KCl, 5 mM MgCl2, 1 mM DTT, 5% glycerol, 0.1% Triton X-100) supplemented with Protease Inhibitor Cocktail (Roche). T7-driven sgRNA was in vitro transcribed using custom oligos (Example 10) and HiScribe T7 In Vitro Transcription Kit (NEB), following the manufacturer's recommended protocol. To prepare methylated target sites, pUC19 plasmid was methylated by M.SssI and then linearized by NheI. The in vitro cleavage assay was performed as follows: for a 20 uL cleavage reaction, 10 uL of cell lysate with incubated with 2 uL cleavage buffer (100 mM HEPES, 500 mM KCl, 25 mM MgCl2, 5 mM DTT, 25% glycerol), the in vitro transcribed RNA, and 300 ng pUC19 plasmid DNA.

[0348] Deep Sequencing to Assess Targeting Specificity

[0349] HEK 293FT cells plated in 96-well plates were transfected with Cas9 plasmid DNA and single guide RNA (sgRNA) PCR cassette 72 hours prior to genomic DNA extraction (FIG. **72**). The genomic region flanking the CRISPR target site for each gene was amplified (FIG. **74**, FIG. **80**, (Example 10) by a fusion PCR method to attach the Illumina P5 adapters as well as unique sample-specific barcodes to the target amplicons (schematic described in FIG. **73**). PCR products were purified using EconoSpin 96-well Filter Plates (Epoch Life Sciences) following the manufacturer's recommended protocol.

[0350] Barcoded and purified DNA samples were quantified by Quant-iT PicoGreen dsDNA Assay Kit or Qubit 2.0 Fluorometer (Life Technologies) and pooled in an equimolar ratio. Sequencing libraries were then deep sequenced with the Illumina MiSeq Personal Sequencer (Life Technologies).

[0351] Sequencing Data Analysis and Indel Detection

[0352] MiSeq reads were filtered by requiring an average Phred quality (Q score) of at least 23, as well as perfect sequence matches to barcodes and amplicon forward primers. Reads from on- and off-target loci were analyzed by first performing Smith-Waterman alignments against amplicon sequences that included 50 nucleotides upstream and downstream of the target site (a total of 120 bp). Alignments, meanwhile, were analyzed for indels from 5 nucleotides upstream to 5 nucleotides downstream of the target site (a total of 30 bp). Analyzed target regions were discarded if part of their alignment fell outside the MiSeq read itself, or if matched base-pairs comprised less than 85% of their total length.

[0353] Negative controls for each sample provided a gauge for the inclusion or exclusion of indels as putative cutting events. For each sample, an indel was counted only if its quality score exceeded $\mu-\sigma$, where μ was the mean quality-score of the negative control corresponding to that sample and σ was the standard deviation of same. This yielded whole target-region indel rates for both negative controls and their corresponding samples. Using the negative control's per-target-region-per-read error rate, q, the sample's observed indel count n, and its read-count R, a maximum-likelihood estimate for the fraction of reads having target-regions with true-indels, P, was derived by applying a binomial error model, as follows.

[0354] Letting the (unknown) number of reads in a sample having target regions incorrectly counted as having at least 1 indel be E, we can write (without making any assumptions about the number of true indels)

$$Prob(E \mid p) = \binom{R(1-p)}{E} q^{E}(1-q)^{R(1-p)-E}$$

[0355] since R(1−p) is the number of reads having target-regions with no true indels. Meanwhile, because the number of reads observed to have indels is n, n=E+Rp, in other words the number of reads having target-regions with errors but no true indels plus the number of reads whose target-regions correctly have indels. We can then re-write the above

$$Prob(E \mid p) = Prob(n = E + Rp \mid p) = \binom{R(1-p)}{n-Rp} q^{n-Rp}(1-q)^{R-n}$$

[0356] Taking all values of the frequency of target-regions with true-indels p to be equally probable a priori, Prob(n|p) αProb(p|n). The maximum-likelihood estimate (MLE) for the frequency of target regions with true-indels was therefore set as the value of p that maximized Prob(n|p). This was evaluated numerically.

[0357] In order to place error bounds on the true-indel read frequencies in the sequencing libraries themselves, Wilson score intervals (2) were calculated for each sample, given the MLE-estimate for true-indel target-regions, Rp, and the number of reads R. Explicitly, the lower bound l and upper bound u were calculated as

$$l = \left(Rp + \frac{z^2}{2} - z\sqrt{Rp(1-p) + z^2/4}\right) \Big/ (R + z^2)$$

[0358] where Z, the standard score for the confidence required in normal distribution of variance 1, was set to 1.96, meaning a confidence of 95%. The maximum upper bounds and minimum lower bounds for each biological replicate are listed in FIGS. **80-83**.

[0359] qRT-PCR Analysis of Relative Cas9 and sgRNA Expression

[0360] 293FT cells plated in 24-well plates were transfected as described above. 72 hours post-transfection, total RNA was harvested with miRNeasy Micro Kit (Qiagen). Reverse-strand synthesis for sgRNAs was performed with qScript Flex cDNA kit (VWR) and custom first-strand synthesis primers (Tables J and K). qPCR analysis was performed with Fast SYBR Green Master Mix (Life Technologies) and custom primers (Tables J and K), using GAPDH as an endogenous control. Relative quantification was calculated by the ΔΔCT method.

TABLE I

Target site sequences. Tested target sites for *S. pyogenes* type II CRISPR system with the requisite PAM. Cells were transfected with Cas9 and either crRNA-tracrRNA or chimeric sgRNA for each target.

Target site ID	genomic target	Target site sequence (5' to 3')	PAM	strand
1	EMX1	GTCACCTCCAATGACTAGGG (SEQ ID NO: 319)	TGG	+
2	EMX1	GACATCGATGTCCTCCCCAT (SEQ ID NO: 196)	TGG	-

TABLE I-continued

Target site sequences. Tested target sites for *S. pyogenes* type II CRISPR system with the requisite PAM. Cells were transfected with Cas9 and either crRNA-tracrRNA or chimeric sgRNA for each target.

Target site ID	genomic target	Target site sequence (5' to 3')	PAM	strand
3	EMX1	GAGTCCGAGCAGAAGAAGAA (SEQ ID NO: 197)	GGG	+
6	EMX1	GCGCCACCGGTTGATGTGAT (SEQ ID NO: 198)	GGG	-
10	EMX1	GGGGCACAGATGAGAAACTC (SEQ ID NO: 199)	AGG	-
11	EMX1	GTACAAACGGCAGAAGCTGG (SEQ ID NO: 200)	AGG	+
12	EMX1	GGCAGAAGCTGGAGGAGGAA (SEQ ID NO: 201)	GGG	+
13	EMX1	GGAGCCCTTCTTCTTCTGCT (SEQ ID NO: 202)	CGG	-
14	EMX1	GGGCAACCACAAACCCACGA (SEQ ID NO: 203)	GGG	+
15	EMX1	GCTCCCATCACATCAACCGG (SEQ ID NO: 204)	TGG	+
16	EMX1	GTGGCGCATTGCCACGAAGC (SEQ ID NO: 205)	AGG	+
17	EMX1	GGCAGAGTGCTGCTTGCTGC (SEQ ID NO: 206)	TGG	+
18	EMX1	GCCCCTGCGTGGGCCCAAGC (SEQ ID NO: 207)	TGG	+
19	EMX1	GAGTGGCCAGAGTCCAGCTT (SEQ ID NO: 208)	GGG	-
20	EMX1	GGCCTCCCCAAAGCCTGGCC (SEQ ID NO: 209)	AGG	-
4	PVALB	GGGGCCGAGATTGGGTGTTC (SEQ ID NO: 210)	AGG	+
5	PVALB	GTGGCGAGAGGGGCCGAGAT (SEQ ID NO: 211)	TGG	+
1	SERPINB5	GAGTGCCGCCGAGGCGGGGC (SEQ ID NO: 212)	GGG	+
2	SERPINB5	GGAGTGCCGCCGAGGCGGGG (SEQ ID NO: 213)	CGG	+
3	SERPINB5	GGAGAGGAGTGCCGCCGAGG (SEQ ID NO: 214)	CGG	+

TABLE J

Primer sequences

SURVEYOR assay

primer name	genomic target	primer sequence (5' to 3')
Sp-EMX1-F1	EMX1	AAAACCACCCTTCTCTCTGGC (SEQ ID NO: 36)
Sp-EMX1-R1	EMX1	GGAGATTGGAGACACGGAGAG (SEQ ID NO: 37)
Sp-EMX1-F2	EMX1	CCATCCCCTTCTGTGAATGT (SEQ ID NO: 215)
Sp-EMX1-R2	EMX1	GGAGATTGGAGACACGGAGA (SEQ ID NO: 216)
Sp-PVALB-F	PVALB	CTGGAAAGCCAATGCCTGAC (SEQ ID NO: 38)
Sp-PVALB-R	PVALB	GGCAGCAAACTCCTTGTCCT (SEQ ID NO: 39)

primer name	primer sequence (5' to 3')

qRT-PCR for Cas9 and sgRNA expression

sgRNA reverse-strand synthesis	AAGCACCGACTCGGTGCCAC (SEQ ID NO: 217)
EMX1.1 sgRNA qPCR F	TCACCTCCAATGACTAGGGG (SEQ ID NO: 218)
EMX1.1 sgRNA qPCR R	CAAGTTGATAACGGACTAGCCT (SEQ ID NO: 219)
EMX1.3 sgRNA qPCR F	AGTCCGAGCAGAAGAAGAAGTTT (SEQ ID NO: 220)
EMX1.3 sgRNA qPCR R	TTTCAAGTTGATAACGGACTAGCCT (SEQ ID NO: 221)
Cas9 qPCR F	AAACAGCAGATTCGCCTGGA (SEQ ID NO: 222)
Cas9 qPCR R	TCATCCGCTCGATGAAGCTC (SEQ ID NO: 223)
GAPDH qPCR F	TCCAAAATCAAGTGGGGCGA (SEQ ID NO: 224)
GAPDH qPCR R	TGATGACCCTTTTGGCTCCC (SEQ ID NO: 225)

Bisulfite PCR and sequencing

Bisulfite PCR F (SERPINB5 locus)	GAGGAATTCTTTTTTTGTTYGAATATGTTGGAGGTTTTTTGGAAG (SEQ ID NO: 226)
Bisulfite PCR R (SERPINB5 locus)	GAGAAGCTTAAATAAAAAACRACAATACTCAACCCAACAACC (SEQ ID NO: 227)
pUC19 sequencing	CAGGAAACAGCTATGAC (SEQ ID NO: 228)

TABLE K

Sequences for primers to test sgRNA architecture. Primers hybridize to the reverse strand of the U6 promoter unless otherwise indicated. The U6 priming site is in italics, the guide sequence is indicated as a stretch of Ns, the direct repeat sequence is highlighted in bold, and the tracrRNA sequence underlined. The secondary structure of each sgRNA architecture is shown in FIG. 43.

primer name	primer sequence (5' to 3')
U6-Forward	GCCTCTA*GAGGTACCTGAGGGCCTATTTCCCATGATTCC* (SEQ ID NO: 229)
I: sgRNA(DR +12, tracrRNA +85)	ACCTCTAGAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTC**TAGCTCTAAAAC**NNNNNNNNNNNNNNNNNNNN*GGTGTTTCGTCCTTTCCACAAG* (SEQ ID NO: 230)
II: sgRNA(DR +12, tracrRNA +85) mut2	ACCTCTAGAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATATTAACTTGCTATTTC**TAGCTCTAATAC**NNNNNNNNNNNNNNNNNNNN*GGTGTTTCGTCCTTTCCACAAG* (SEQ ID NO: 231)
III: sgRNA(DR +22, tracrRNA +85)	ACCTCTAGAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATGCTGTTTTGTTTCC**CAAAACAGCATAGCTCTAAAAC**NNNNNNNNNNNNNNNNNNNN*GGTGTTTCGTCCTTTCCACAAG* (SEQ ID NO: 232)
IV: sgRNA(DR +22, tracrRNA +85) mut4	ACCTCTAGAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATATTAACTTGCTATGCTGTATTGTTTC**CAATACAGCATAGCTCTAATAC**NNNNNNNNNNNNNNNNNNNN*GGTGTTTCGTCCTTTCCACAAG* (SEQ ID NO: 233)

TABLE L

Target sites with alternate PAMs for testing PAM specificity of Cas9. All target sites for PAM specificity testing are found within the human EMX1 locus.

Target site sequence (5' to 3')	PAM
AGGCCCCAGTGGCTGCTCT (SEQ ID NO: 234)	NAA
ACATCAACCGGTGGCGCAT (SEQ ID NO: 235)	NAT
AAGGTGTGGTTCCAGAACC (SEQ ID NO: 236)	NAC
CCATCACATCAACCGGTGG (SEQ ID NO: 237)	NAG
AAACGGCAGAAGCTGGAGG (SEQ ID NO: 238)	NTA
GGCAGAAGCTGGAGGAGGA (SEQ ID NO: 239)	NTT
GGTGTGGTTCCAGAACCGG (SEQ ID NO: 240)	NTC
AACCGGAGGACAAAGTACA (SEQ ID NO: 241)	NTG
TTCCAGAACCGGAGGACAA (SEQ ID NO: 242)	NCA
GTGTGGTTCCAGAACCGGA (SEQ ID NO: 243)	NCT
TCCAGAACCGGAGGACAAA (SEQ ID NO: 244)	NCC
CAGAAGCTGGAGGAGGAAG (SEQ ID NO: 245)	NCG
CATCAACCGGTGGCGCATT (SEQ ID NO: 246)	NGA
GCAGAAGCTGGAGGAGGAA (SEQ ID NO: 247)	NGT
CCTCCCTCCCTGGCCCAGG (SEQ ID NO: 248)	NGC
TCATCTGTGCCCCTCCCTC (SEQ ID NO: 249)	NAA
GGGAGGACATCGATGTCAC (SEQ ID NO: 250)	NAT

TABLE L-continued

Target sites with alternate PAMs for testing PAM specificity of Cas9. All target sites for PAM specificity testing are found within the human EMX1 locus.

Target site sequence (5' to 3')	PAM
CAAACGGCAGAAGCTGGAG (SEQ ID NO: 251)	NAC
GGGTGGGCAACCACAAACC (SEQ ID NO: 252)	NAG
GGTGGGCAACCACAAACCC (SEQ ID NO: 253)	NTA
GGCTCCCATCACATCAACC (SEQ ID NO: 254)	NTT
GAAGGGCCTGAGTCCGAGC (SEQ ID NO: 255)	NTC
CAACCGGTGGCGCATTGCC (SEQ ID NO: 256)	NTG
AGGAGGAAGGGCCTGAGTC (SEQ ID NO: 257)	NCA
AGCTGGAGGAGGAAGGGCC (SEQ ID NO: 258)	NCT
GCATTGCCACGAAGCAGGC (SEQ ID NO: 259)	NCC
ATTGCCACGAAGCAGGCCA (SEQ ID NO: 260)	NCG
AGAACCGGAGGACAAAGTA (SEQ ID NO: 261)	NGA
TCAACCGGTGGCGCATTGC (SEQ ID NO: 262)	NGT
GAAGCTGGAGGAGGAAGGG (SEQ ID NO: 263)	NGC

Example 10

Supplementary Sequences

[0361] All sequences are in the 5' to 3' direction. For U6 transcription, the string of underlined Ts serve as the transcriptional terminator.

> U6-short tracrRNA (*Streptococcus pyogenes* SF370)
(SEQ ID NO: 40)

gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagag
agataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtga
cgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggact
atcatatgcttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaa
ggacgaaacacc**GGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAG**
TCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT

(tracrRNA sequence in bold)
>U6-DR-guide sequence-DR (*Streptococcus pyogenes* SF370)
(SEQ ID NO: 54)

gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagaga
gataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacg
tagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatc
atatgcttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggac
gaaacaccgggttttagagctatgctgttttgaatggtcccaaaac**NNNNNNNN**
NNNNNNNNNNNNNNNNNNNNgttttagagctatgctgttttgaatggt
cccaaaacTTTTTTT

(direct repeat sequence is highlighted in gray and the guide sequence is in bold Ns)
> sgRNA containing +48 tracrRNA (*Streptococcus pyogenes* SF370)
(SEQ ID NO: 55)

gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagaga
taattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaa
agtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgc
ttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaac

-continued

acc**NNNNNNNNNNNNNNNNNNNN**gttttagagctagaaa**tagcaagtta**
aaataaggctagtccgTTTTTTT

(guide sequence is in bold Ns and the tracrRNA fragment
is in bold)
> sgRNA containing +54 tracrRNA (*Streptococcus pyogenes* SF370)
(SEQ ID NO: 56)
gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagag
ataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgta
gaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgc
ttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaaca
cc**NNNNNNNNNNNNNNNNNNNN**gttttagagctagaaa**tagcaagttaaa**
ataaggctagtccgttatcaTTTTTTTT

(guide sequence is in bold Ns and the tracrRNA fragment
is in bold)
> sgRNA containing +67 tracrRNA (*Streptococcus pyogenes* SF370)
(SEQ ID NO: 57)
gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagaga
taattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtaga
aagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgc
ttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaac
acc**NNNNNNNNNNNNNNNNNNNN**gttttagagctagaaa**tagcaagtta**
aaataaggctagtccgttatcaacttgaaaaagtgTTTTTTT

(guide sequence is in bold Ns and the tracrRNA fragment
is in bold)
> sgRNA containing +85 tracrRNA (*Streptococcus pyogenes* SF370)
(SEQ ID NO: 58)
gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagag
ataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtag
aaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgc
ttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaaca
cc**NNNNNNNNNNNNNNNNNNNN**gttttagagctagaaa**tagcaagttaa**
aataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcTTTTTTT

(guide sequence is in bold Ns and the tracrRNA fragment
is in bold)
> CBh-NLS-SpCas9-NLS
(SEQ ID NO: 59)
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACC
CCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTT
TCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATC
AAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCG
CCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTA
CGTATTAGTCATCGCTATTACCATGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTC
CCCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATTTATTTTTTAATTATTTTG
TGCAGCGATGGGGGCGGGGGGGGGGGGGCGCGCCAGGCGGGGCGGGGCGG
GGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCG
GCGCGCTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGCCCTATAAA
AAGCGAAGCGCGCGGCGGGCGGGAGTCGCTGCGACGCTGCCTTCGCCCCGTGCCCC
GCTCCGCCGCCGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGTTACTCCCAC
AGGTGAGCGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATTAGCTGAGCAAGAGG
TAAGGGTTTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATTACCTGGAGCAC
CTGCCTGAAATCACTTTTTTTCAGGTTGGaccggtgccacc**ATGGACTATAAGGACCACG**
ACGGAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATAAGATG
GCCCCAAAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCCCAGCAGCCGACAA
GAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTGA
TCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACC
GACCGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGG
CGAAACAGCCGAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACCA
GACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGCAACGAGATGGCC
AAGGTGGACGACAGCTTCTTCCACAGACTGGAAGAGTCCTTCCTGGTGGAAGA
GGATAAGAAGCACGAGCGGCACCCCATCTTCGGCAACATCGTGGACGAGGTGG
CCTACCACGAGAAGTACCCCACCATCTACCACCTGAGAAAGAAACTGGTGGAC
AGCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCACATGAT
CAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTGAACCCCGACAACAGCG
ACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAG
GAAAACCCCATCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAG
ACTGAGCAAGAGCAGACGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGAGA
AGAAGAATGGCCTGTTCGGCAACCTGATTGCCCTGAGCCTGGGCCTGACCCCC
AACTTCAAGAGCAACTTCGACCTGGCCGAGGATGCCAAACTGCAGCTGAGCAA
GGACACCTACGACGACGACCTGGACAACCTGCTGGCCCAGATCGGCGACCAGT
ACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATCCTGCTGAGC
GACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCTAT
GATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCG
TGCGGCAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAG
AACGGCTACGCCGGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTTCTACAA

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GTTCATCAAGCCCATCCTGGAAAAGATGGACGGCACCGAGGAACTGCTCGTGA
AGCTGAACAGAGAGGACCTGCTGCGGAAGCAGCGGACCTTCGACAACGGCAG
CATCCCCCACCAGATCCACCTGGGAGAGCTGCACGCCATTCTGCGGCGGCAGG
AAGATTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATCGAGAAGATCCTG
ACCTTCCGCATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAGCAGATT
CGCCTGGATGACCAGAAAGAGCGAGGAAACCATCACCCCCTGGAACTTCGAGG
AAGTGGTGGACAAGGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCAAC
TTCGATAAGAACCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTA
CGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATACGTGACCGAGG
GAATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAGGCCATCGTGGAC
CTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGAAAGAGGACTA
CTTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATCTCCGGCGTGGAAGATC
GGTTCAACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATTATCAAGGAC
AAGGACTTCCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTGCT
GACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCT
ATGCCCACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATAC
ACCGGCTGGGGCAGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGC
AGTCCGGCAAGACAATCCTGGATTTCCTGAAGTCCGACGGCTTCGCCAACAGA
AACTTCATGCAGCTGATCCACGACGACAGCCTGACCTTTAAAGAGGACATCCA
GAAAGCCCAGGTGTCCGGCCAGGGCGATAGCCTGCACGAGCACATTGCCAATC
TGGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACAGTGAAGGTGGTG
GACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGAGAACATCGTGATCGA
AATGGCCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGAACAGCCGCGAG
AGAATGAAGCGGATCGAAGAGGGCATCAAAGAGCTGGGCAGCCAGATCCTGAA
AGAACACCCCGTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACT
ACCTGCAGAATGGGCGGGATATGTACGTGGACCAGGAACTGGACATCAACCGG
CTGTCCGACTACGATGTGGACCATATCGTGCCTCAGAGCTTTCTGAAGGACGA
CTCCATCGACAACAAGGTGCTGACCAGAAGCGACAAGAACCGGGGCAAGAGCG
ACAACGTGCCCTCCGAAGAGGTCGTGAAGAAGATGAAGAACTACTGGCGGCAG
CTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTGACCAAGGC
CGAGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAG
CTGGTGGAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCCG
GATGAACACTAAGTACGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGA
TCACCCTGAAGTCCAAGCTGGTGTCCGATTTCCGGAAGGATTTCCAGTTTTACA
AAGTGCGCGAGATCAACAACTACCACCACGCCCACGACGCCTACCTGAACGCC
GTCGTGGGAACCGCCCTGATCAAAAAGTACCCTAAGCTGGAAAGCGAGTTCGT
GTACGGCGACTACAAGGTGTACGACGTGCGGAAGATGATCGCCAAGAGCGAGC
AGGAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGCAACATCATGAACT
TTTTCAAGACCGAGATTACCCTGGCCAACGGCGAGATCCGGAAGCGGCCTCTG
ATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGATT
TTGCCACCGTGCGGAAAGTGCTGAGCATGCCCCAAGTGAATATCGTGAAAAAG
ACCGAGGTGCAGACAGGCGGCTTCAGCAAAGAGTCTATCCTGCCCAAGAGGAA
CAGCGATAAGCTGATCGCCAGAAAGAAGGACTGGGACCCTAAGAAGTACGGCG
GCTTCGACAGCCCCACCGTGGCCTATTCTGTGCTGGTGGTGGCCAAAGTGGAA
AAGGGCAAGTCCAAGAAACTGAAGAGTGTGAAAGAGCTGCTGGGGATCACCAT
CATGGAAAGAAGCAGCTTCGAGAAGAATCCCATCGACTTTCTGGAAGCCAAGG
GCTACAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCCTAAGTACTCCCTG
TTCGAGCTGGAAAACGGCCGGAAGAGAATGCTGGCCTCTGCCGGCGAACTGCA
GAAGGGAAACGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTGG
CCAGCCACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAACAG
CTGTTTGTGGAACAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAG
CGAGTTCTCCAAGAGAGTGATCCTGGCCGACGCTAATCTGGACAAAGTGCTGT
CCGCCTACAACAAGCACCGGGATAAGCCCATCAGAGAGCAGGCCGAGAATATC
ATCCACCTGTTTACCCTGACCAATCTGGGAGCCCCTGCCGCCTTCAAGTACTTT
GACACCACCATCGACCGGAAGAGGTACACCAGCACCAAAGAGGTGCTGGACGC
CACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATCGACCTGT
CTCAGCTGGGAGGCGACTTTCTTTTTCTTAGCTTGACCAGCTTTCTTAGTAGCA
GCAGGACGCTTTAA

(NLS-hSpCas9-NLS is highlighted in bold)

> Sequencing amplicon for EMX1 guides 1.1, 1.14, 1.17

(SEQ ID NO: 264)

CCAATGGGGAGGACATCGATGTCACCTCCAATGACTAGGGTGGGCAACC
ACAAACCCACGAGGGCAGAGTGCTGCTTGCTGCTGGCCAGGCCCCTGCGTGGGCCC
AAGCTGGACTCTGGCCAC

> Sequencing amplicon for EMX1 guides 1.2, 1.16

(SEQ ID NO: 265)

CGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCC
ACGAAGCAGGCCAATGGGGAGGACATCGATGTCACCTCCAATGACTAGGGTGGGCA
ACCACAAACCCACGAG

> Sequencing amplicon for EMX1 guides 1.3, 1.13, 1.15

(SEQ ID NO: 266)

GGAGGACAAAGTACAAACGGCAGAAGCTGGAGGAGGAAGGGCCTGAGTC
CGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCACGAAGC
AGGCCAATGGGGAGGACATCGAT

-continued

> Sequencing amplicon for EMX1 guides 1.6
(SEQ ID NO: 267)
AGAAGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTC
CCATCACATCAACCGGTGGCGCATTGCCACGAAGCAGGCCAATGGGGAGGACATCG
ATGTCACCTCCAATGACTAGGGTGG

> Sequencing amplicon for EMX1 guides 1.10
(SEQ ID NO: 268)
CCTCAGTCTTCCCATCAGGCTCTCAGCTCAGCCTGAGTGTTGAGGCCCCAG
TGGCTGCTCTGGGGGCCTCCTGAGTTTCTCATCTGTGCCCCTCCCTCCCTGGCCCAGG
TGAAGGTGTGGTTCCA

> Sequencing amplicon for EMX1 guides 1.11, 1.12
(SEQ ID NO: 269)
TCATCTGTGCCCCTCCCTCCCTGGCCCAGGTGAAGGTGTGGTTCCAGAACC
GGAGGACAAAGTACAAACGGCAGAAGCTGGAGGAGGAAGGGCCTGAGTCCGAGCA
GAAGAAGAAGGGCTCCCATCACA

> Sequencing amplicon for EMX1 guides 1.18, 1.19
(SEQ ID NO: 270)
CTCCAATGACTAGGGTGGGCAACCACAAACCCACGAGGGCAGAGTGCTG
CTTGCTGCTGGCCAGGCCCCTGCGTGGGCCCAAGCTGGACTCTGGCCACTCCCTGGC
CAGGCTTTGGGGAGGCCTGGAGT

> Sequencing amplicon for EMX1 guides 1.20
(SEQ ID NO: 271)
CTGCTTGCTGCTGGCCAGGCCCCTGCGTGGGCCCAAGCTGGACTCTGGCC
ACTCCCTGGCCAGGCTTTGGGGAGGCCTGGAGTCATGGCCCCACAGGGCTTGAAGC
CCGGGGCCGCCATTGACAGAG

>T7 promoter F primer for annealing with target strand
(SEQ ID NO: 272)
GAAATTAATACGACTCACTATAGGG

>oligo containing pUC19 target site 1 for methylation (T7 reverse)
(SEQ ID NO: 273)
AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCC
TTATTTTAACTTGCTATTTCTAGCTCTAAAACAACGACGAGCGTGACACCACCCTAT
AGTGAGTCGTATTAATTTC

>oligo containing pUC19 target site 2 for methylation (T7 reverse)
(SEQ ID NO: 274)
AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCC
TTATTTTAACTTGCTATTTCTAGCTCTAAAACGCAACAATTAATAGACTGGACCTATA
GTGAGTCGTATTAATTTC

Example 11

Oligo-Mediated Cas9-Induced Homologous Recombination

[0362] The oligo homologous recombination test is a comparison of efficiency across different Cas9 variants and different HR template (oligo vs. plasmid).

[0363] 293FT cells were used. SpCas9=Wildtype Cas9 and SpCas9n=nickase Cas9 (D10A). The chimeric RNA target is the same EMX1 Protospacer Target 1 as in Examples 5, 9 and 10 and oligos synthesized by IDT using PAGE purification.

[0364] FIG. **44** depicts a design of the oligo DNA used as Homologous Recombination (HR) template in this experiment. Long oligos contain 100 bp homology to the EMX1 locus and a HindIII restriction site. 293FT cells were co-transfected with: first, a plasmid containing a chimeric RNA targeting human EMX1 locus and wild-type cas9 protein, and second, the oligo DNA as HR template. Samples are from 293FT cells collected 96 hours post transfection with Lipofectamine 2000. All products were amplified with an EMX1 HR Primer, gel purified, followed by digestion with HindIII to detect the efficiency of integration of HR template into the human genome.

[0365] FIGS. **45** and **46** depict a comparison of HR efficiency induced by different combination of Cas9 protein and HR template. The Cas9 construct used were either wild-type Cas9 or the nickase version of Cas9 (Cas9n). The HR template used were: antisense oligo DNA (Antisense-Oligo in above figure), or sense oligo DNA (Sense-Oligo in above figure), or plasmid HR template (HR template in above figure). The sense/anti-sense definition is that the actively-transcribed strand with sequence corresponding to the transcribed mRNA is defined as the sense strand of genome. HR Efficiency is shown as percentage of HindIII digestion band as against all genomic PCR amplified product (bottom numbers).

Example 12

Autistic Mouse

[0366] Recent large-scale sequencing initiatives have produced a large number of genes associated with disease. Discovering the genes is only the beginning in understanding what the gene does and how it leads to a diseased phenotype. Current technologies and approaches to study candidate genes are slow and laborious. The gold standards, gene targeting and genetic knockouts, require a significant investment in time and resources, both monetary and in terms of research personnel. Applicants set out to utilize the hSpCas9 nuclease to target many genes and do so with higher effi-

ciency and lower turnaround compared to any other technology. Because of the high efficiency of hSpCas9 Applicants can do RNA injection into mouse zygotes and immediately get genome-modified animals without the need to do any preliminary gene targeting in mESCs.

[0367] Chromodomain helicase DNA binding protein 8 (CHD8) is a pivotal gene in involved in early vertebrate development and morphogenesis. Mice lacking CHD8 die during embryonic development. Mutations in the CHD8 gene have been associated with autism spectrum disorder in humans. This association was made in three different papers published simultaneously in Nature. The same three studies identified a plethora of genes associated with autism spectrum disorder. Applicants' aim was to create knockout mice for the four genes that were found in all papers, Chd8, Katna12, Kctd13, and Scn2a. In addition, Applicants chose two other genes associated with autism spectrum disorder, schizophrenia, and ADHD, GIT1, CACNA1C, and CACNB2. And finally, as a positive control Applicants decide to target MeCP2.

[0368] For each gene Applicants designed three gRNAs that would likely knockout the gene. A knockout would occur after the hSpCas9 nuclease makes a double strand break and the error prone DNA repair pathway, non-homologous end joining, corrects the break, creating a mutation. The most likely result is a frameshift mutation that would knockout the gene. The targeting strategy involved finding proto-spacers in the exons of the gene that had a PAM sequence, NGG, and was unique in the genome. Preference was given to proto-spacers in the first exon, which would be most deleterious to the gene.

[0369] Each gRNA was validated in the mouse cell line, Neuro-N2a, by liposomal transient co-transfection with hSpCas9. 72 hours post-transfection genomic DNA was purified using QuickExtract DNA from Epicentre. PCR was performed to amplify the locus of interest. Subsequently the SURVEYOR Mutation Detection Kit from Transgenomics was followed. The SURVEYOR results for each gRNA and respective controls are shown in Figure **A1**. A positive SURVEYOR result is one large band corresponding to the genomic PCR and two smaller bands that are the product of the SURVEYOR nuclease making a double-strand break at the site of a mutation. The average cutting efficiency of each gRNA was also determined for each gRNA. The gRNA that was chosen for injection was the highest efficiency gRNA that was the most unique within the genome.

[0370] RNA (hSpCas9+gRNA RNA) was injected into the pronucleus of a zygote and later transplanted into a foster mother. Mothers were allowed to go full term and pups were sampled by tail snip 10 days postnatal. DNA was extracted and used as a template for PCR, which was then processed by SURVEYOR. Additionally, PCR products were sent for sequencing. Animals that were detected as being positive in either the SURVEYOR assay or PCR sequencing would have their genomic PCR products cloned into a pUC 19 vector and sequenced to determine putative mutations from each allele.

[0371] So far, mice pups from the Chd8 targeting experiment have been fully processed up to the point of allele sequencing. The Surveyor results for 38 live pups (lanes 1-38) 1 dead pup (lane 39) and 1 wild-type pup for comparison (lane 40) are shown in Figure **A2**. Pups 1-19 were injected with gRNA Chd8.2 and pups 20-38 were injected with gRNA Chd8.3. Of the 38 live pups, 13 were positive for a mutation. The one dead pup also had a mutation. There was no mutation detected in the wild-type sample. Genomic PCR sequencing was consistent with the SURVEYOR assay findings.

Example 13

CRISPR/Cas-Mediated Transcriptional Modulation

[0372] FIG. **67** depicts a design of the CRISPR-TF (Transcription Factor) with transcriptional activation activity. The chimeric RNA is expressed by U6 promoter, while a human-codon-optimized, double-mutant version of the Cas9 protein (hSpCas9m), operably linked to triple NLS and a VP64 functional domain is expressed by a EF1a promoter. The double mutations, D10A and H840A, renders the cas9 protein unable to introduce any cleavage but maintained its capacity to bind to target DNA when guided by the chimeric RNA.

[0373] FIG. **68** depicts transcriptional activation of the human SOX2 gene with CRISPR-TF system (Chimeric RNA and the Cas9-NLS-VP64 fusion protein). 293FT cells were transfected with plasmids bearing two components: (1) U6-driven different chimeric RNAs targeting 20-bp sequences within or around the human SOX2 genomic locus, and (2) EF1a-driven hSpCas9m (double mutant)-NLS-VP64 fusion protein. 96 hours post transfection, 293FT cells were harvested and the level of activation is measured by the induction of mRNA expression using a qRT-PCR assay. All expression levels are normalized against the control group (grey bar), which represents results from cells transfected with the CRISPR-TF backbone plasmid without chimeric RNA. The qRT-PCR probes used for detecting the SOX2 mRNA is Taqman Human Gene Expression Assay (Life Technologies). All experiments represents data from 3 biological replicates, n=3, error bars show s.e.m.

Example 14

NLS: Cas9 NLS

[0374] 293FT cells were transfected with plasmid containing two components: (1) EF1a promoter driving the expression of Cas9 (wild-type human-codon-optimized Sp Cas9) with different NLS designs (2) U6 promoter driving the same chimeric RNA targeting human EMX1 locus.

[0375] Cells were collect at 72 h time point post transfection, and then extracted with 50 μl of the QuickExtract genomic DNA extraction solution following manufacturer's protocol. Target EMX1 genomic DNA were PCR amplified and then Gel-purify with 1% agarose gel. Genomic PCR product were re-anneal and subjected to the Surveyor assay following manufacturer's protocol. The genomic cleavage efficiency of different constructs were measured using SDS-PAGE on a 4-12% TBE-PAGE gel (Life Technologies), analyzed and quantified with ImageLab (Bio-rad) software, all following manufacturer's protocol.

[0376] FIG. **69** depicts a design of different Cas9 NLS constructs. All Cas9 were the human-codon-optimized version of the Sp Cas9. NLS sequences are linked to the cas9 gene at either N-terminus or C-terminus. All Cas9 variants with different NLS designs were cloned into a backbone vector containing so it is driven by EF1a promoter. On the same vector there is a chimeric RNA targeting human EMX1 locus driven by U6 promoter, together forming a two-component system.

TABLE M

Cas9 NLS Design Test Results. Quantification of genomic cleavage of different cas9-nls constructs by surveyor assay.

Percentage Genome Cleavage as measured by Surveyor assay	Biological Replicate 1 (%)	Biological Replicate 2 (%)	Biological Replicate 3 (%)	Average (%)	Error (S.E.M., standard error of the mean)
Cas9 (No NLS)	2.50	3.30	2.73	2.84	0.24
Cas9 with N-term NLS	7.61	6.29	5.46	6.45	0.63
Cas9 with C-term NLS	5.75	4.86	4.70	5.10	0.33
Cas9 with Double (N-term and C-term) NLS	9.08	9.85	7.78	8.90	0.60

[0377] FIG. **70** depicts the efficiency of genomic cleavage induced by Cas9 variants bearing different NLS designs. The percentage indicate the portion of human EMX1 genomic DNA that were cleaved by each construct. All experiments are from 3 biological replicates. n 3, error indicates S.E.M.

Example 15

Engineering of Microalgae using Cas9

[0378] Methods of Delivering Cas9

[0379] Method 1: Applicants deliver Cas9 and guide RNA using a vector that expresses Cas9 under the control of a constitutive promoter such as Hsp70A-Rbc S2 or Beta2-tubulin.

[0380] Method 2: Applicants deliver Cas9 and T7 polymerase using vectors that expresses Cas9 and T7 polymerase under the control of a constitutive promoter such as Hsp70A-Rbc S2 or Beta2-tubulin. Guide RNA will be delivered using a vector containing T7 promoter driving the guide RNA.

[0381] Method 3: Applicants deliver Cas9 mRNA and in vitro transcribed guide RNA to algae cells. RNA can be in vitro transcribed. Cas9 mRNA will consist of the coding region for Cas9 as well as 3'UTR from Cop1 to ensure stabilization of the Cas9 mRNA.

[0382] For Homologous recombination, Applicants provide an additional homology directed repair template.

[0383] Sequence for a cassette driving the expression of Cas9 under the control of beta-2 tubulin promoter, followed by the 3' UTR of Cop1.

(SEQ ID NO: 275)

TCTTTCTTGCGCTATGACACTTCCAGCAAAAGGTAGGGCGGGCTGCGAGA

CGGCTTCCCGGCGCTGCATGCAACACCGATGATGCTTCGACCCCCCGAAGCTCCTTC

GGGGCTGCATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTTAAATAGCCAG

GCCCCCGATTGCAAAGACATTATAGCGAGCTACCAAAGCCATATTCAAACACCTAG

ATCACTACCACTTCTACACAGGCCACTCGAGCTTGTGATCGCACTCCGCTAAGGGGG

CGCCTCTTCCTCTTCGTTTCAGTCACAACCCGCAAACATGTACCCATACGATGTTCCA

GATTACGCTTCGCCGAAGAAAAAGCGCAAGGTCGAAGCGTCCGACAAGAAGTACAG

CATCGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTGATCACCGACGAGT

ACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGGCACAGCATC

AAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAGGCCAC

CCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGC

TATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCAC

AGACTGGAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCCAT

CTTCGGCAACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCCACCATCTACC

ACCTGAGAAAGAAACTGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGATCTAT

CTGGCCCTGGCCCACATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTG

AACCCCGACAACAGCGACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAA

CCAGCTGTTCGAGGAAAACCCCATCAACGCCAGCGGCGTGGACGCCAAGGCCATCC

-continued

TGTCTGCCAGACTGAGCAAGAGCAGACGGCTGGAAAATCTGATCGCCCAGCTGCCC

GGCGAGAAGAAGAATGGCCTGTTCGGCAACCTGATTGCCCTGAGCCTGGGCCTGAC

CCCCAACTTCAAGAGCAACTTCGACCTGGCCGAGGATGCCAAACTGCAGCTGAGCA

AGGACACCTACGACGACGACCTGGACAACCTGCTGGCCCAGATCGGCGACCAGTAC

GCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATCCTGCTGAGCGACATC

CTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCTATGATCAAGAG

ATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCGTGCGGCAGCAGC

TGCCTGAGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCCGGC

TACATTGACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCT

GGAAAAGATGGACGGCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTG

CTGCGGAAGCAGCGGACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCTGGG

AGAGCTGCACGCCATTCTGCGGCGGCAGGAAGATTTTTACCCATTCCTGAAGGACAA

CCGGGAAAAGATCGAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGCCCTCT

GGCCAGGGGAAACAGCAGATTCGCCTGGATGACCAGAAAGAGCGAGGAAACCATC

ACCCCCTGGAACTTCGAGGAAGTGGTGGACAAGGGCGCTTCCGCCCAGAGCTTCAT

CGAGCGGATGACCAACTTCGATAAGAACCTGCCCAACGAGAAGGTGCTGCCCAAGC

ACAGCCTGCTGTACGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATAC

GTGACCGAGGGAATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAGGCCA

TCGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGAAAGAG

GACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATCTCCGGCGTGGAAGAT

CGGTTCAACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATTATCAAGGACAA

GGACTTCCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTGCTGACCC

TGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCCCAC

CTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGG

CAGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACA

ATCCTGGATTTCCTGAAGTCCGACGGCTTCGCCAACAGAAACTTCATGCAGCTGATC

CACGACGACAGCCTGACCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGGCCA

GGGCGATAGCCTGCACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATTAAGA

AGGGCATCCTGCAGACAGTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGGCCGG

CACAAGCCCGAGAACATCGTGATCGAAATGGCCAGAGAGAACCAGACCACCCAGA

AGGGACAGAAGAACAGCCGCGAGAGAATGAAGCGGATCGAAGAGGGCATCAAAGA

GCTGGGCAGCCAGATCCTGAAAGAACACCCCGTGGAAAACACCCAGCTGCAGAACG

AGAAGCTGTACCTGTACTACCTGCAGAATGGGCGGGATATGTACGTGGACCAGGAA

CTGGACATCAACCGGCTGTCCGACTACGATGTGGACCATATCGTGCCTCAGAGCTTT

CTGAAGGACGACTCCATCGACAACAAGGTGCTGACCAGAAGCGACAAGAACCGGG

GCAAGAGCGACAACGTGCCCTCCGAAGAGGTCGTGAAGAAGATGAAGAACTACTGG

CGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTGACCAA

GGCCGAGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAG

CTGGTGGAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCCGGAT

-continued

GAACACTAAGTACGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATCACCC

TGAAGTCCAAGCTGGTGTCCGATTTCCGGAAGGATTTCCAGTTTTACAAAGTGCGCG

AGATCAACAACTACCACCACGCCCACGACGCCTACCTGAACGCCGTCGTGGGAACC

GCCCTGATCAAAAAGTACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAA

GGTGTACGACGTGCGGAAGATGATCGCCAAGAGCGAGCAGGAAATCGGCAAGGCT

ACCGCCAAGTACTTCTTCTACAGCAACATCATGAACTTTTTCAAGACCGAGATTACC

CTGGCCAACGGCGAGATCCGGAAGCGGCCTCTGATCGAGACAAACGGCGAAACCGG

GGAGATCGTGTGGGATAAGGGCCGGGATTTTGCCACCGTGCGGAAAGTGCTGAGCA

TGCCCCAAGTGAATATCGTGAAAAAGACCGAGGTGCAGACAGGCGGCTTCAGCAAA

GAGTCTATCCTGCCCAAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGGACTG

GGACCCTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGTGCTGGT

GGTGGCCAAAGTGGAAAAGGGCAAGTCCAAGAAACTGAAGAGTGTGAAAGAGCTG

CTGGGGATCACCATCATGGAAAGAAGCAGCTTCGAGAAGAATCCCATCGACTTTCT

GGAAGCCAAGGGCTACAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCCTAAGT

ACTCCCTGTTCGAGCTGGAAAACGGCCGGAAGAGAATGCTGGCCTCTGCCGGCGAA

CTGCAGAAGGGAAACGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTG

GCCAGCCACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAACAGCT

GTTTGTGGAACAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGT

TCTCCAAGAGAGTGATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACA

ACAAGCACCGGGATAAGCCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTT

ACCCTGACCAATCTGGGAGCCCCTGCCGCCTTCAAGTACTTTGACACCACCATCGAC

CGGAAGAGGTACACCAGCACCAAAGAGGTGCTGGACGCCACCCTGATCCACCAGAG

CATCACCGGCCTGTACGAGACACGGATCGACCTGTCTCAGCTGGGAGGCGACAGCC

CCAAGAAGAAGAGAAAGGTGGAGGCCAGCTAAGGATCCGGCAAGACTGGCCCCGC

TTGGCAACGCAACAGTGAGCCCCTCCCTAGTGTGTTTGGGGATGTGACTATGTATTC

GTGTGTTGGCCAACGGGTCAACCCGAACAGATTGATACCCGCCTTGGCATTTCCTGT

CAGAATGTAACGTCAGTTGATGGTACT

[0384] Sequence for a cassette driving the expression of T7 polymerase under the control of beta-2 tubulin promoter, followed by the 3' UTR of Cop1:

(SEQ ID NO: 276)

TCTTTCTTGCGCTATGACACTTCCAGCAAAAGGTAGGGCGGGCTGCGAGA

CGGCTTCCCGGCGCTGCATGCAACACCGATGATGCTTCGACCCCCCGAAGCTCCTTC

GGGGCTGCATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTTAAATAGCCAG

GCCCCCGATTGCAAAGACATTATAGCGAGCTACCAAAGCCATATTCAAACACCTAG

ATCACTACCACTTCTACACAGGCCACTCGAGCTTGTGATCGCACTCCGCTAAGGGGG

CGCCTCTTCCTCTTCGTTTCAGTCACAACCCGCAAACatgcctaagaagaagaggaaggttaacacgatt

aacatcgctaagaacgacttctctgacatcgaactggctgctatcccgttcaacactctggctgaccattacggtgagcgtttagctcgcgaa

cagttggcccttgagcatgagtcttacgagatgggtgaagcacgcttccgcaagatgtttgagcgtcaacttaaagctggtgaggttgcggat

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aacgctgccgccaagcctctcatcactaccctactccctaagatgattgcacgcatcaacgactggtttgaggaagtgaaagctaagcgcg

gcaagcgcccgacagccttccagttcctgcaagaaatcaagccggaagccgtagcgtacatcaccattaagaccactctggcttgcctaac

cagtgctgacaatacaaccgttcaggctgtagcaagcgcaatcggtcgggccattgaggacgaggctcgcttcggtcgtatccgtgacctt

gaagctaagcacttcaagaaaaacgttgaggaacaactcaacaagcgcgtagggcacgtctacaagaaagcatttatgcaagttgtcgag

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atacgctgaggctatcgcaacccgtgcaggtgcgctggctggcatctctccgatgttccaaccttgcgtagttcctcctaagccgtggactgg

cattactggtggtggctattgggctaacggtcgtcgtcctctggcgctggtgcgtactcacagtaagaaagcactgatgcgctacgaagacg

tttacatgcctgaggtgtacaaagcgattaacattgcgcaaaacaccgcatggaaaatcaacaagaaagtcctagcggtcgccaacgtaatc

accaagtggaagcattgtccggtcgaggacatccctgcgattgagcgtgaagaactcccgatgaaaccggaagacatcgacatgaatcct

gaggctctcaccgcgtggaaacgtgctgccgctgctgtgtaccgcaaggacaaggctcgcaagtctcgccgtatcagccttgagttcatgc

ttgagcaagccaataagtttgctaaccataaggccatctggttcccttacaacatggactggcgcggtcgtgtttacgctgtgtcaatgttcaac

ccgcaaggtaacgatatgaccaaaggactgcttacgctggcgaaaggtaaaccaatcggtaaggaaggttactactggctgaaaatccac

ggtgcaaactgtgcgggtgtcgacaaggttccgttccctgagcgcatcaagttcattgaggaaaaccacgagaacatcatggcttgcgctaa

gtctccactggagaacacttggtgggctgagcaagattctccgttctgcttccttgcgttctgctttgagtacgctggggtacagcaccacggc

ctgagctataactgctcccttccgctggcgtttgacgggtcttgctctggcatccagcacttctccgcgatgctccgagatgaggtaggtggtc

gcgcggttaacttgcttcctagtgaaaccgttcaggacatctacgggattgttgctaagaaagtcaacgagattctacaagcagacgcaatca

atgggaccgataacgaagtagttaccgtgaccgatgagaacactggtgaaatctctgagaaagtcaagctgggcactaaggcactggctg

gtcaatggctggcttacggtgttactcgcagtgtgactaagcgttcagtcatgacgctggcttacgggtccaaagagttcggcttccgtcaac

aagtgctggaagataccattcagccagctattgattccggcaagggtctgatgttcactcagccgaatcaggctgctggatacatggctaag

ctgatttgggaatctgtgagcgtgacggtggtagctgcggttgaagcaatgaactggcttaagtctgctgctaagctgctggctgctgaggtc

aaagataagaagactggagagattcttcgcaagcgttgcgctgtgcattgggtaactcctgatggtttccctgtgtggcaggaatacaagaa

gcctattcagacgcgcttgaacctgatgttcctcggtcagttccgcttacagcctaccattaacaccaacaaagatagcgagattgatgcaca

caaacaggagtctggtatcgctcctaactttgtacacagccaagacggtagccaccttcgtaagactgtagtgtgggcacacgagaagtac

ggaatcgaatcttttgcactgattcacgactccttcggtacgattccggctgacgctgcgaacctgttcaaagcagtgcgcgaaactatggttg

acacatatgagtcttgtgatgtactggctgatttctacgaccagttcgctgaccagttgcacgagtctcaattggacaaaatgccagcacttcc

ggctaaaggtaacttgaacctccgtgacatcttagagtcggacttcgcgttcgcgtaaGGATCCGGCAAGACTGGCCCC

GCTTGGCAACGCAACAGTGAGCCCCTCCCTAGTGTGTTTGGGGATGTGACTATGTAT

TCGTGTGTTGGCCAACGGGTCAACCCGAACAGATTGATACCCGCCTTGGCATTTCCT

GTCAGAATGTAACGTCAGTTGATGGTACT

[0385] Sequence of guide RNA driven by the T7 promoter (T7 promoter, Ns represent targeting sequence):

(SEQ ID NO: 277)

gaaat**TAATACGACTCACTATA**NNNNNNNNNNNNNNNNNNNgttttag

agctaGAAAtagcaagttaaaataaggctagtccgttatcaacttgaaa

aagtggcaccgagtcggtgctttttttt

[0386] Gene Delivery:

[0387] *Chlamydomonas reinhardtii* strain CC-124 and CC-125 from the *Chlamydomonas* Resource Center will be used for electroporation. Electroporation protocol follows standard recommended protocol from the GeneArt *Chlamydomonas* Engineering kit.

[0388] Also, Applicants generate a line of *Chlamydomonas reinhardtii* that expresses Cas9 constitutively. This can be done by using pChlamy1 (linearized using PvuI) and selecting for hygromycin resistant colonies. Sequence for pChlamy1 containing Cas9 is below. In this way to achieve gene knockout one simply needs to deliver RNA for the guideRNA. For homologous recombination Applicants deliver guideRNA as well as a linearized homologous recombination template.

pChlamyl-Cas9:

(SEQ ID NO: 278)

TGCGGTATTTCACACCGCATCAGGTGGCACTTTTCGGGGAAATGTGCGCG

GAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGATT

ATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAAT

CTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGC

ACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTG

TAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCG

CGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAG

GGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTG

TTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGC

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CTAGTGTGTTTGGGGATGTGACTATGTATTCGTGTGTTGGCCAACGGGTCAACCCGA

ACAGATTGATACCCGCCTTGGCATTTCCTGTCAGAATGTAACGTCAGTTGATGGTACT

[0389] For all modified *Chlamydomonas* reinhardtii cells, Applicants used PCR, SURVEYOR nuclease assay, and DNA sequencing to verify successful modification.

Example 16

Use of Cas9 as a Transcriptional Repressor in Bacteria

[0390] The ability to artificially control transcription is essential both to the study of gene function and to the construction of synthetic gene networks with desired properties. Applicants describe here the use of the RNA-guided Cas9 protein as a programmable transcriptional repressor.

[0391] Applicants have previously demonstrated how the Cas9 protein of *Streptococcus pyogenes* SF370 can be used to direct genome editing in *Streptococcus pneumoniae*. In this study Applicants engineered the crR6Rk strain containing a minimal CRISPR system, consisting of cas9, the tracrRNA and a repeat. The D10A-H840 mutations were introduced into cas9 in this strain, giving strain crR6Rk**. Four spacers targeting different positions of the bgaA β-galactosidase gene promoter were cloned in the CRISPR array carried by the previously described pDB98 plasmid. Applicants observed a X to Y fold reduction in β-galactosidase activity depending on the targeted position, demonstrating the potential of Cas9 as a programmable repressor (FIG. **73**).

[0392] To achieve Cas9** repression in *Escherichia coli* a green fluorescence protein (GFP) reporter plasimd (pDB127) was constructred to express the gfpmut2 gene from a constituitive promoter. The promoter was designed to carry several NPP PAMs on both strands, to measure the effect of Cas9** binding at various positions. Applicants introduced the D10A-H840 mutations into pCas9, a plasmid described carrying the tracrRNA, cas9 and a minimal CRISPR array designed for the easy cloning of new spacers. Twenty-two different spacers were designed to target different regions of the gfpmut2 promoter and open reading frame. An approximately 20-fold reduction of fluorescence of was observed upon targeting regions overlapping or adjacent to the −35 and −10 promoter elements and to the Shine-Dalgarno sequence. Targets on both strands showed similar repression levels. These results suggest that the binding of Cas9** to any position of the promoter region prevents transcription initiation, presumably through steric inhibition of RNAP binding.

[0393] To determine whether Cas9** could prevent transcription elongation, Applicants directed it to the reading frame of gpfmut2. A reduction in fluorescence was observed both when the coding and non-coding strands where targeted, suggesting that Cas9 binding is actually strong enough to represent an obstacle to the running RNAP. However, while a 40% reduction in expression was observed when the coding strand was the target, a 20-fold reduction was observed for the non-coding strand (FIG. **21***b*, compare T9, T10 and T11 to B9, B10 and B 11). To directly determine the effects of Cas9** binding on transcription, Applicants extracted RNA from strains carrying either the T5, T10, B 10 or a control construct that does not target pDB127 and subjected it to Northern blot analysis using either a probe binding before (B477) or after (B510) the B10 and T10 target sites. Consistent with Applicants' fluorescence methods, no gfpmut2 transcription was detected when Cas9** was directed to the promoter region (T5 target) and a transcription was observed after the targeting of the T10 region. Interestingly, a smaller transcript was observed with the B477 probe. This band corresponds to the expected size of a transcript that would be interrupted by Cas9**, and is a direct indication of a transcriptional termination caused by dgRNA::Cas9** binding to the coding strand. Surprisingly, Applicants detected no transcript when the non-coding strand was targeted (B10). Since Cas9** binding to the B 10 region is unlikely to interfere with transcription initiation, this result suggests that the mRNA was degraded. DgRNA::Cas9 was shown to bind ssRNA in vitro. Applicants speculate that binding may trigger degradation of the mRNA by host nucleases. Indeed, ribosome stalling can induce cleavage on the translated mRNA in *E. coli.*

[0394] Some applications require a precise tuning gene expression rather than its complete repression. Applicants sought to achieve intermediate repression levels through the introduction of mismatches that will weaken the crRNA/target interactions. Applicants created a series of spacers based on the B 1, T5 and B 10 constructs with increasing numbers of mutations in the 5' end of the crRNA. Up to 8 mutations in B1 and T5 did not affect the repression level, and a progressive increased in fluorescence was observed for additional mutations.

[0395] The observed repression with only an 8 nt match between the crRNA and its target raises the question of off-targeting effects of the use of Cas9** as a transcriptional regulator. Since a good PAM (NGG) is also required for Cas9 binding, the number of nucleotides to match to obtain some level of respiration is 10. A 10 nt match occurs randomly once every ~1 Mbp, and such sites are thus likely to be found even in small bacterial genomes. However, to effectively repress transcription, such site needs to be in the promoter region of gene, which makes off-targeting much less likely. Applicants also showed that gene expression can be affected if the non-coding strand of a gene is targeted. For this to happen, a random target would have to be in the right orientation, but such events relatively more likely to happen. As a matter of fact, during the course of this study Applicants were unable to construct one of the designed spacer on pCas9**. Applicants later found this spacer showed a 12 bp match next to a good PAM in the essential murC gene. Such off-targeting could easily be avoided by a systematic blast of the designed spacers.

[0396] Aspects of the invention are further described in the following numbered paragraphs:

[0397] 1. A vector system comprising one or more vectors, wherein the system comprises

[0398] a. a first regulatory element operably linked to a traer mate sequence and one or more insertion sites for inserting a guide sequence upstream of the traer mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the traer mate sequence that is hybridized to the traer sequence; and

[0399] b. a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence;

[0400] wherein components (a) and (b) are located on the same or different vectors of the system.

[0401] 2. The vector system of paragraph 1, wherein component (a) further comprises the traer sequence downstream of the traer mate sequence under the control of the first regulatory element.

[0402] 3. The vector system of paragraph 1, wherein component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell.

[0403] 4. The vector system of paragraph 1, wherein the system comprises the traer sequence under the control of a third regulatory element.

[0404] 5. The vector system of paragraph 1, wherein the traer sequence exhibits at least 50% of sequence complementarity along the length of the traer mate sequence when optimally aligned.

[0405] 6. The vector system of paragraph 1, wherein the CRISPR enzyme comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell.

[0406] 7. The vector system of paragraph 1, wherein the CRISPR enzyme is a type II CRISPR system enzyme.

[0407] 8. The vector system of paragraph 1, wherein the CRISPR enzyme is a Cas9 enzyme.

[0408] 9. The vector system of paragraph 1, wherein the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell.

[0409] 10. The vector system of paragraph 1, wherein the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence.

[0410] 11. The vector system of paragraph 1, wherein the CRISPR enzyme lacks DNA strand cleavage activity.

[0411] 12. The vector system of paragraph 1, wherein the first regulatory element is a polymerase III promoter.

[0412] 13. The vector system of paragraph 1, wherein the second regulatory element is a polymerase II promoter.

[0413] 14. The vector system of paragraph 4, wherein the third regulatory element is a polymerase III promoter.

[0414] 15. The vector system of paragraph 1, wherein the guide sequence is at least 15 nucleotides in length.

[0415] 16. The vector system of paragraph 1, wherein fewer than 50% of the nucleotides of the guide sequence participate in self-complementary base-pairing when optimally folded.

[0416] 17. A vector comprising a regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising one or more nuclear localization sequences, wherein said regulatory element drives transcription of the CRISPR enzyme in a eukaryotic cell such that said CRISPR enzyme accumulates in a detectable amount in the nucleus of the eukaryotic cell.

[0417] 18. The vector of paragraph 17, wherein said regulatory element is a polymerase II promoter.

[0418] 19. The vector of paragraph 17, wherein said CRISPR enzyme is a type IICRISPR system enzyme.

[0419] 20. The vector of paragraph 17, wherein said CRISPR enzyme is a Cas9 enzyme.

[0420] 21. The vector of paragraph 17, wherein said CRISPR enzyme lacks the ability to cleave one or more strands of a target sequence to which it binds.

[0421] 22. A CRISPR enzyme comprising one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell.

[0422] 23. The CRISPR enzyme of paragraph 22, wherein said CRISPR enzyme is a type IICRISPR system enzyme.

[0423] 24. The CRISPR enzyme of paragraph 22, wherein said CRISPR enzyme is a Cas9 enzyme.

[0424] 25. The CRISPR enzyme of paragraph 22, wherein said CRISPR enzyme lacks the ability to cleave one or more strands of a target sequence to which it binds.

[0425] 26. A eukaryotic host cell comprising:

[0426] a. a first regulatory element operably linked to a traer mate sequence and one or more insertion sites for inserting a guide sequence upstream of the traer mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the traer mate sequence that is hybridized to the traer sequence; and/or

[0427] b. a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence.

[0428] 27. The eukaryotic host cell of paragraph 26, wherein said host cell comprises components (a) and (b).

[0429] 28. The eukaryotic host cell of paragraph 26, wherein component (a), component (b), or components (a) and (b) are stably integrated into a genome of the host eukaryotic cell.

[0430] 29. The eukaryotic host cell of paragraph 26, wherein component (a) further comprises the traer sequence downstream of the traer mate sequence under the control of the first regulatory element.

[0431] 30. The eukaryotic host cell of paragraph 26, wherein component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell.

[0432] 31. The eukaryotic host cell of paragraph 26, further comprising a third regulatory element operably linked to said traer sequence.

[0433] 32. The eukaryotic host cell of paragraph 26, wherein the traer sequence exhibits at least 50% of sequence complementarity along the length of the traer mate sequence when optimally aligned.

[0434] 33. The eukaryotic host cell of paragraph 26, wherein the CRISPR enzyme comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable mount in the nucleus of a eukaryotic cell.

[0435] 34. The eukaryotic host cell of paragraph 26, wherein the CRISPR enzyme is a type II CRISPR system enzyme.

[0436] 35. The eukaryotic host cell of paragraph 26, wherein the CRISPR enzyme is a Cas9 enzyme.

[0437] 36. The eukaryotic host cell of paragraph 26, wherein the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell.

[0438] 37. The eukaryotic host cell of paragraph 26, wherein the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence.

[0439] 38. The eukaryotic host cell of paragraph 26, wherein the CRISPR enzyme lacks DNA strand cleavage activity.

[0440] 39. The eukaryotic host cell of paragraph 26, wherein the first regulatory element is a polymerase III promoter.

[0441] 40. The eukaryotic host cell of paragraph 26, wherein the second regulatory element is a polymerase II promoter.

[0442] 41. The eukaryotic host cell of paragraph 31, wherein the third regulatory element is a polymerase III promoter.

[0443] 42. The eukaryotic host cell of paragraph 26, wherein the guide sequence is at least 15 nucleotides in length.

[0444] 43. The eukaryotic host cell of paragraph 26, wherein fewer than 50% of the nucleotides of the guide sequence participate in self-complementary base-pairing when optimally folded.

[0445] 44. A non-human animal comprising a eukaryotic host cell of any one of paragraphs 26-43.

[0446] 45. A kit comprising a vector system and instructions for using said kit, the vector system comprising:

[0447] a. a first regulatory element operably linked to a traer mate sequence and one or more insertion sites for inserting a guide sequence upstream of the traer mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the traer mate sequence that is hybridized to the traer sequence; and/or

[0448] b. a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence.

[0449] 46. The kit of paragraph 45, wherein said kit comprises components (a) and (b) located on the same or different vectors of the system.

[0450] 47. The kit of paragraph 45, wherein component (a) further comprises the traer sequence downstream of the traer mate sequence under the control of the first regulatory element.

[0451] 48. The kit of paragraph 45, wherein component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell.

[0452] 49. The kit of paragraph 45, wherein the system comprises the traer sequence under the control of a third regulatory element.

[0453] 50. The kit of paragraph 45, wherein the traer sequence exhibits at least 50% of sequence complementarity along the length of the traer mate sequence when optimally aligned.

[0454] 51. The kit of paragraph 45, wherein the CRISPR enzyme comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable mount in the nucleus of a eukaryotic cell.

[0455] 52. The kit of paragraph 45, wherein the CRISPR enzyme is a type II CRISPR system enzyme.

[0456] 53. The kit of paragraph 45, wherein the CRISPR enzyme is a Cas9 enzyme.

[0457] 54. The kit of paragraph 45, wherein the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell.

[0458] 55. The kit of paragraph 45, wherein the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence.

[0459] 56. The kit of paragraph 45, wherein the CRISPR enzyme lacks DNA strand cleavage activity.

[0460] 57. The kit of paragraph 45, wherein the first regulatory element is a polymerase III promoter.

[0461] 58. The kit of paragraph 45, wherein the second regulatory element is a polymerase II promoter.

[0462] 59. The kit of paragraph 49, wherein the third regulatory element is a polymerase III promoter.

[0463] 60. The kit of paragraph 45, wherein the guide sequence is at least 15 nucleotides in length.

[0464] 61. The kit of paragraph 45, wherein fewer than 50% of the nucleotides of the guide sequence participate in self-complementary base-pairing when optimally folded.

[0465] 62. A computer system for selecting a candidate target sequence within a nucleic acid sequence in a eukaryotic cell for targeting by a CRISPR complex, the system comprising:

[0466] a. a memory unit configured to receive and/or store said nucleic acid sequence; and

[0467] b. one or more processors alone or in combination programmed to (i) locate a CRISPR motif sequence within said nucleic acid sequence, and (ii) select a sequence adjacent to said located CRISPR motif sequence as the candidate target sequence to which the CRISPR complex binds.

[0468] 63. The computer system of paragraph 62, wherein said locating step comprises identifying a CRISPR motif sequence located less than about 500 nucleotides away from said target sequence.

[0469] 64. The computer system of paragraph 62, wherein said candidate target sequence is at least 10 nucleotides in length.

[0470] 65. The computer system of paragraph 62, wherein the nucleotide at the 3' end of the candidate target sequence is located no more than about 10 nucleotides upstream of the CRISPR motif sequence.

[0471] 66. The computer system of paragraph 62, wherein the nucleic acid sequence in the eukaryotic cell is endogenous to the eukaryotic genome.

[0472] 67. The computer system of claim **62**, wherein the nucleic acid sequence in the eukaryotic cell is exogenous to the eukaryotic genome.

[0473] 68. A computer-readable medium comprising codes that, upon execution by one or more processors, implements a method of selecting a candidate target sequence within a nucleic acid sequence in a eukaryotic cell for targeting by a CRISPR complex, said method comprising:

[0474] (a) locating a CRISPR motif sequence within said nucleic acid sequence, and (b) selecting a sequence adjacent to said located CRISPR motif sequence as the candidate target sequence to which the CRISPR complex binds.

[0475] 69. The computer-readable medium of paragraph 68, wherein said locating comprises locating a CRISPR motif sequence that is less than about 500 nucleotides away from said target sequence.

[0476] 70. The computer-readable of paragraph 68, wherein said candidate target sequence is at least 10 nucleotides in length.

[0477] 71. The computer-readable of paragraph 68, wherein the nucleotide at the 3' end of the candidate target sequence is located no more than about 10 nucleotides upstream of the CRISPR motif sequence.

[0478] 72. The computer-readable of paragraph 68, wherein the nucleic acid sequence in the eukaryotic cell is endogenous the eukaryotic genome.

[0479] 73. The computer-readable of paragraph 68, wherein the nucleic acid sequence in the eukaryotic cell is exogenous to the eukaryotic genome.

[0480] 74. A method of modifying a target polynucleotide in a eukaryotic cell, the method comprising allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a traer mate sequence which in turn hybridizes to a traer sequence.

[0481] 75. The method of paragraph 74, wherein said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme.

[0482] 76. The method of paragraph 74, wherein said cleavage results in decreased transcription of a target gene.

[0483] 77. The method of paragraph 74, further comprising repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide.

[0484] 78. The method of paragraph 77, wherein said mutation results in one or more amino acid changes in a protein expressed from a gene comprising the target sequence.

[0485] 79. The method of paragraph 74, further comprising delivering one or more vectors to said eukaryotic cell, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the traer mate sequence, and the traer sequence.

[0486] 80. The method of paragraph 79, wherein said vectors are delivered to the eukaryotic cell in a subject.

[0487] 81. The method of paragraph 74, wherein said modifying takes place in said eukaryotic cell in a cell culture.

[0488] 82. The method of paragraph 74, further comprising isolating said eukaryotic cell from a subject prior to said modifying.

[0489] 83. The method of paragraph 82, further comprising returning said eukaryotic cell and/or cells derived therefrom to said subject.

[0490] 84. A method of modifying expression of a polynucleotide in a eukaryotic cell, the method comprising: allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a traer mate sequence which in turn hybridizes to a traer sequence.

[0491] 85. The method of paragraph 74, further comprising delivering one or more vectors to said eukaryotic cells, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the traer mate sequence, and the traer sequence.

[0492] 86. A method of generating a model eukaryotic cell comprising a mutated disease gene, the method comprising:

[0493] a. introducing one or more vectors into a eukaryotic cell, wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a traer mate sequence, and a traer sequence; and

[0494] b. allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said disease gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the traer mate sequence that is hybridized to the traer sequence, thereby generating a model eukaryotic cell comprising a mutated disease gene.

[0495] 87. The method of paragraph 86, wherein said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme.

[0496] 88. The method of paragraph 86, wherein said cleavage results in decreased transcription of a target gene.

[0497] 89. The method of paragraph 86, further comprising repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide.

[0498] 90. The method of paragraph 89, wherein said mutation results in one or more amino acid changes in a protein expressed from a gene comprising the target sequence.

[0499] 91. A method of developing a biologically active agent that modulates a cell signaling event associated with a disease gene, comprising:

[0500] a. contacting a test compound with a model cell of any one of paragraphs 86-90; and

[0501] b. detecting a change in a readout that is indicative of a reduction or an augmentation of a cell signaling event associated with said mutation in said disease gene, thereby developing said biologically active agent that modulates said cell signaling event associated with said disease gene.

[0502] 92. A recombinant polynucleotide comprising a guide sequence upstream of a traer mate sequence, wherein the guide sequence when expressed directs sequence-specific binding of a CRISPR complex to a corresponding target sequence present in a eukaryotic cell.

[0503] 93. The recombinant polynucleotide of paragraph 89, wherein the target sequence is a viral sequence present in a eukaryotic cell.

[0504] 94. The recombinant polynucleotide of paragraph 89, wherein the target sequence is a proto-oncogene or an oncogene.

[0505] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 33

ggaagggcct gagtccgagc agaagaagaa ggg 33

<210> SEQ ID NO 34
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 34

ggtggcgaga ggggccgaga ttgggtgttc agg 33

<210> SEQ ID NO 35
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 35

atgcaggagg gtggcgagag gggccgagat tgg 33

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<210> SEQ ID NO 36
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 36

aaaaccaccc ttctctctgg c 21

<210> SEQ ID NO 37
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 37

ggagattgga gacacggaga g 21

<210> SEQ ID NO 38
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 38

ctggaaagcc aatgcctgac 20

<210> SEQ ID NO 39
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 39

ggcagcaaac tccttgtcct 20

<210> SEQ ID NO 40
<211> LENGTH: 335
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 40

gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc tgttagagag 60

ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga 120

aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccg gaaccattca aaacagcata gcaagttaaa ataaggctag tccgttatca 300

acttgaaaaa gtggcaccga gtcggtgctt ttttt 335

<210> SEQ ID NO 41

-continued

<211> LENGTH: 423
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 41

gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc tgttagagag 60

ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga 120

aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccg gtagtattaa gtattgtttt atggctgata aatttctttg aatttctcct 300

tgattatttg ttataaaagt tataaaataa tcttgttgga accattcaaa acagcatagc 360

aagttaaaat aaggctagtc cgttatcaac ttgaaaaagt ggcaccgagt cggtgctttt 420

ttt 423

<210> SEQ ID NO 42
<211> LENGTH: 339
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 42

gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc tgttagagag 60

ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga 120

aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccg ggttttagag ctatgctgtt ttgaatggtc ccaaaacggg tcttcgagaa 300

gacgttttag agctatgctg ttttgaatgg tcccaaaac 339

<210> SEQ ID NO 43
<211> LENGTH: 309
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 43

gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc tgttagagag 60

ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga 120

aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccg ggtcttcgag aagacctgtt ttagagctag aaatagcaag ttaaaataag 300

gctagtccg 309

<210> SEQ ID NO 44
<211> LENGTH: 1648
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polypeptide

<400> SEQUENCE: 44

```
Met Asp Tyr Lys Asp His Asp Gly Asp Tyr Lys Asp His Asp Ile Asp
1               5                  10                  15

Tyr Lys Asp Asp Asp Asp Lys Met Ala Pro Lys Lys Lys Arg Lys Val
            20                  25                  30

Gly Ile His Gly Val Pro Ala Ala Asp Lys Lys Tyr Ser Ile Gly Leu
        35                  40                  45

Asp Ile Gly Thr Asn Ser Val Gly Trp Ala Val Ile Thr Asp Glu Tyr
    50                  55                  60

Lys Val Pro Ser Lys Lys Phe Lys Val Leu Gly Asn Thr Asp Arg His
65                  70                  75                  80

Ser Ile Lys Lys Asn Leu Ile Gly Ala Leu Leu Phe Asp Ser Gly Glu
                85                  90                  95

Thr Ala Glu Ala Thr Arg Leu Lys Arg Thr Ala Arg Arg Arg Tyr Thr
            100                 105                 110

Arg Arg Lys Asn Arg Ile Cys Tyr Leu Gln Glu Ile Phe Ser Asn Glu
        115                 120                 125

Met Ala Lys Val Asp Asp Ser Phe Phe His Arg Leu Glu Glu Ser Phe
    130                 135                 140

Leu Val Glu Glu Asp Lys Lys His Glu Arg His Pro Ile Phe Gly Asn
145                 150                 155                 160

Ile Val Asp Glu Val Ala Tyr His Glu Lys Tyr Pro Thr Ile Tyr His
                165                 170                 175

Leu Arg Lys Lys Leu Val Asp Ser Thr Asp Lys Ala Asp Leu Arg Leu
            180                 185                 190

Ile Tyr Leu Ala Leu Ala His Met Ile Lys Phe Arg Gly His Phe Leu
        195                 200                 205

Ile Glu Gly Asp Leu Asn Pro Asp Asn Ser Asp Val Asp Lys Leu Phe
    210                 215                 220

Ile Gln Leu Val Gln Thr Tyr Asn Gln Leu Phe Glu Glu Asn Pro Ile
225                 230                 235                 240

Asn Ala Ser Gly Val Asp Ala Lys Ala Ile Leu Ser Ala Arg Leu Ser
                245                 250                 255

Lys Ser Arg Arg Leu Glu Asn Leu Ile Ala Gln Leu Pro Gly Glu Lys
            260                 265                 270

Lys Asn Gly Leu Phe Gly Asn Leu Ile Ala Leu Ser Leu Gly Leu Thr
        275                 280                 285

Pro Asn Phe Lys Ser Asn Phe Asp Leu Ala Glu Asp Ala Lys Leu Gln
    290                 295                 300

Leu Ser Lys Asp Thr Tyr Asp Asp Asp Leu Asp Asn Leu Leu Ala Gln
305                 310                 315                 320

Ile Gly Asp Gln Tyr Ala Asp Leu Phe Leu Ala Ala Lys Asn Leu Ser
                325                 330                 335

Asp Ala Ile Leu Leu Ser Asp Ile Leu Arg Val Asn Thr Glu Ile Thr
            340                 345                 350

Lys Ala Pro Leu Ser Ala Ser Met Ile Lys Arg Tyr Asp Glu His His
        355                 360                 365

Gln Asp Leu Thr Leu Leu Lys Ala Leu Val Arg Gln Gln Leu Pro Glu
    370                 375                 380
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Lys Tyr Lys Glu Ile Phe Phe Asp Gln Ser Lys Asn Gly Tyr Ala Gly
385                 390                 395                 400

Tyr Ile Asp Gly Gly Ala Ser Gln Glu Glu Phe Tyr Lys Phe Ile Lys
                405                 410                 415

Pro Ile Leu Glu Lys Met Asp Gly Thr Glu Glu Leu Leu Val Lys Leu
            420                 425                 430

Asn Arg Glu Asp Leu Leu Arg Lys Gln Arg Thr Phe Asp Asn Gly Ser
        435                 440                 445

Ile Pro His Gln Ile His Leu Gly Glu Leu His Ala Ile Leu Arg Arg
    450                 455                 460

Gln Glu Asp Phe Tyr Pro Phe Leu Lys Asp Asn Arg Glu Lys Ile Glu
465                 470                 475                 480

Lys Ile Leu Thr Phe Arg Ile Pro Tyr Tyr Val Gly Pro Leu Ala Arg
                485                 490                 495

Gly Asn Ser Arg Phe Ala Trp Met Thr Arg Lys Ser Glu Glu Thr Ile
            500                 505                 510

Thr Pro Trp Asn Phe Glu Glu Val Val Asp Lys Gly Ala Ser Ala Gln
        515                 520                 525

Ser Phe Ile Glu Arg Met Thr Asn Phe Asp Lys Asn Leu Pro Asn Glu
    530                 535                 540

Lys Val Leu Pro Lys His Ser Leu Leu Tyr Glu Tyr Phe Thr Val Tyr
545                 550                 555                 560

Asn Glu Leu Thr Lys Val Lys Tyr Val Thr Glu Gly Met Arg Lys Pro
                565                 570                 575

Ala Phe Leu Ser Gly Glu Gln Lys Lys Ala Ile Val Asp Leu Leu Phe
            580                 585                 590

Lys Thr Asn Arg Lys Val Thr Val Lys Gln Leu Lys Glu Asp Tyr Phe
        595                 600                 605

Lys Lys Ile Glu Cys Phe Asp Ser Val Glu Ile Ser Gly Val Glu Asp
    610                 615                 620

Arg Phe Asn Ala Ser Leu Gly Thr Tyr His Asp Leu Leu Lys Ile Ile
625                 630                 635                 640

Lys Asp Lys Asp Phe Leu Asp Asn Glu Glu Asn Glu Asp Ile Leu Glu
                645                 650                 655

Asp Ile Val Leu Thr Leu Thr Leu Phe Glu Asp Arg Glu Met Ile Glu
            660                 665                 670

Glu Arg Leu Lys Thr Tyr Ala His Leu Phe Asp Asp Lys Val Met Lys
        675                 680                 685

Gln Leu Lys Arg Arg Arg Tyr Thr Gly Trp Gly Arg Leu Ser Arg Lys
    690                 695                 700

Leu Ile Asn Gly Ile Arg Asp Lys Gln Ser Gly Lys Thr Ile Leu Asp
705                 710                 715                 720

Phe Leu Lys Ser Asp Gly Phe Ala Asn Arg Asn Phe Met Gln Leu Ile
                725                 730                 735

His Asp Asp Ser Leu Thr Phe Lys Glu Asp Ile Gln Lys Ala Gln Val
            740                 745                 750

Ser Gly Gln Gly Asp Ser Leu His Glu His Ile Ala Asn Leu Ala Gly
        755                 760                 765

Ser Pro Ala Ile Lys Lys Gly Ile Leu Gln Thr Val Lys Val Val Asp
    770                 775                 780

Glu Leu Val Lys Val Met Gly Arg His Lys Pro Glu Asn Ile Val Ile
785                 790                 795                 800
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Glu Met Ala Arg Glu Asn Gln Thr Thr Gln Lys Gly Gln Lys Asn Ser
                805                 810                 815

Arg Glu Arg Met Lys Arg Ile Glu Glu Gly Ile Lys Glu Leu Gly Ser
            820                 825                 830

Gln Ile Leu Lys Glu His Pro Val Glu Asn Thr Gln Leu Gln Asn Glu
        835                 840                 845

Lys Leu Tyr Leu Tyr Tyr Leu Gln Asn Gly Arg Asp Met Tyr Val Asp
    850                 855                 860

Gln Glu Leu Asp Ile Asn Arg Leu Ser Asp Tyr Asp Val Asp His Ile
865                 870                 875                 880

Val Pro Gln Ser Phe Leu Lys Asp Asp Ser Ile Asp Asn Lys Val Leu
                885                 890                 895

Thr Arg Ser Asp Lys Asn Arg Gly Lys Ser Asp Asn Val Pro Ser Glu
            900                 905                 910

Glu Val Val Lys Lys Met Lys Asn Tyr Trp Arg Gln Leu Leu Asn Ala
        915                 920                 925

Lys Leu Ile Thr Gln Arg Lys Phe Asp Asn Leu Thr Lys Ala Glu Arg
    930                 935                 940

Gly Gly Leu Ser Glu Leu Asp Lys Ala Gly Phe Ile Lys Arg Gln Leu
945                 950                 955                 960

Val Glu Thr Arg Gln Ile Thr Lys His Val Ala Gln Ile Leu Asp Ser
                965                 970                 975

Arg Met Asn Thr Lys Tyr Asp Glu Asn Asp Lys Leu Ile Arg Glu Val
            980                 985                 990

Lys Val Ile Thr Leu Lys Ser Lys  Leu Val Ser Asp Phe  Arg Lys Asp
        995                 1000                1005

Phe Gln  Phe Tyr Lys Val Arg  Glu Ile Asn Asn Tyr  His His Ala
    1010                1015                1020

His Asp  Ala Tyr Leu Asn Ala  Val Val Gly Thr Ala  Leu Ile Lys
    1025                1030                1035

Lys Tyr  Pro Lys Leu Glu Ser  Glu Phe Val Tyr Gly  Asp Tyr Lys
    1040                1045                1050

Val Tyr  Asp Val Arg Lys Met  Ile Ala Lys Ser Glu  Gln Glu Ile
    1055                1060                1065

Gly Lys  Ala Thr Ala Lys Tyr  Phe Phe Tyr Ser Asn  Ile Met Asn
    1070                1075                1080

Phe Phe  Lys Thr Glu Ile Thr  Leu Ala Asn Gly Glu  Ile Arg Lys
    1085                1090                1095

Arg Pro  Leu Ile Glu Thr Asn  Gly Glu Thr Gly Glu  Ile Val Trp
    1100                1105                1110

Asp Lys  Gly Arg Asp Phe Ala  Thr Val Arg Lys Val  Leu Ser Met
    1115                1120                1125

Pro Gln  Val Asn Ile Val Lys  Lys Thr Glu Val Gln  Thr Gly Gly
    1130                1135                1140

Phe Ser  Lys Glu Ser Ile Leu  Pro Lys Arg Asn Ser  Asp Lys Leu
    1145                1150                1155

Ile Ala  Arg Lys Lys Asp Trp  Asp Pro Lys Lys Tyr  Gly Gly Phe
    1160                1165                1170

Asp Ser  Pro Thr Val Ala Tyr  Ser Val Leu Val Val  Ala Lys Val
    1175                1180                1185

Glu Lys  Gly Lys Ser Lys Lys  Leu Lys Ser Val Lys  Glu Leu Leu
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    1190                1195                1200

Gly Ile  Thr Ile Met Glu Arg  Ser Ser Phe Glu Lys  Asn Pro Ile
    1205                1210                1215

Asp Phe  Leu Glu Ala Lys Gly  Tyr Lys Glu Val Lys  Lys Asp Leu
    1220                1225                1230

Ile Ile  Lys Leu Pro Lys Tyr  Ser Leu Phe Glu Leu  Glu Asn Gly
    1235                1240                1245

Arg Lys  Arg Met Leu Ala Ser  Ala Gly Glu Leu Gln  Lys Gly Asn
    1250                1255                1260

Glu Leu  Ala Leu Pro Ser Lys  Tyr Val Asn Phe Leu  Tyr Leu Ala
    1265                1270                1275

Ser His  Tyr Glu Lys Leu Lys  Gly Ser Pro Glu Asp  Asn Glu Gln
    1280                1285                1290

Lys Gln  Leu Phe Val Glu Gln  His Lys His Tyr Leu  Asp Glu Ile
    1295                1300                1305

Ile Glu  Gln Ile Ser Glu Phe  Ser Lys Arg Val Ile  Leu Ala Asp
    1310                1315                1320

Ala Asn  Leu Asp Lys Val Leu  Ser Ala Tyr Asn Lys  His Arg Asp
    1325                1330                1335

Lys Pro  Ile Arg Glu Gln Ala  Glu Asn Ile Ile His  Leu Phe Thr
    1340                1345                1350

Leu Thr  Asn Leu Gly Ala Pro  Ala Ala Phe Lys Tyr  Phe Asp Thr
    1355                1360                1365

Thr Ile  Asp Arg Lys Arg Tyr  Thr Ser Thr Lys Glu  Val Leu Asp
    1370                1375                1380

Ala Thr  Leu Ile His Gln Ser  Ile Thr Gly Leu Tyr  Glu Thr Arg
    1385                1390                1395

Ile Asp  Leu Ser Gln Leu Gly  Gly Asp Ala Ala Ala  Val Ser Lys
    1400                1405                1410

Gly Glu  Glu Leu Phe Thr Gly  Val Val Pro Ile Leu  Val Glu Leu
    1415                1420                1425

Asp Gly  Asp Val Asn Gly His  Lys Phe Ser Val Ser  Gly Glu Gly
    1430                1435                1440

Glu Gly  Asp Ala Thr Tyr Gly  Lys Leu Thr Leu Lys  Phe Ile Cys
    1445                1450                1455

Thr Thr  Gly Lys Leu Pro Val  Pro Trp Pro Thr Leu  Val Thr Thr
    1460                1465                1470

Leu Thr  Tyr Gly Val Gln Cys  Phe Ser Arg Tyr Pro  Asp His Met
    1475                1480                1485

Lys Gln  His Asp Phe Phe Lys  Ser Ala Met Pro Glu  Gly Tyr Val
    1490                1495                1500

Gln Glu  Arg Thr Ile Phe Phe  Lys Asp Asp Gly Asn  Tyr Lys Thr
    1505                1510                1515

Arg Ala  Glu Val Lys Phe Glu  Gly Asp Thr Leu Val  Asn Arg Ile
    1520                1525                1530

Glu Leu  Lys Gly Ile Asp Phe  Lys Glu Asp Gly Asn  Ile Leu Gly
    1535                1540                1545

His Lys  Leu Glu Tyr Asn Tyr  Asn Ser His Asn Val  Tyr Ile Met
    1550                1555                1560

Ala Asp  Lys Gln Lys Asn Gly  Ile Lys Val Asn Phe  Lys Ile Arg
    1565                1570                1575
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His Asn  Ile Glu Asp Gly Ser  Val Gln Leu Ala Asp  His Tyr Gln
    1580                1585                1590

Gln Asn  Thr Pro Ile Gly Asp  Gly Pro Val Leu Leu  Pro Asp Asn
    1595                1600                1605

His Tyr  Leu Ser Thr Gln Ser  Ala Leu Ser Lys Asp  Pro Asn Glu
    1610                1615                1620

Lys Arg  Asp His Met Val Leu  Leu Glu Phe Val Thr  Ala Ala Gly
    1625                1630                1635

Ile Thr  Leu Gly Met Asp Glu  Leu Tyr Lys
    1640                1645


<210> SEQ ID NO 45
<211> LENGTH: 1625
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polypeptide

<400> SEQUENCE: 45

Met Asp Lys Lys Tyr Ser Ile Gly Leu Asp Ile Gly Thr Asn Ser Val
1               5                   10                  15

Gly Trp Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe
            20                  25                  30

Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile
        35                  40                  45

Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
    50                  55                  60

Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
65                  70                  75                  80

Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser
                85                  90                  95

Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
            100                 105                 110

His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr
        115                 120                 125

His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp
    130                 135                 140

Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
145                 150                 155                 160

Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
                165                 170                 175

Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr
            180                 185                 190

Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp Ala
        195                 200                 205

Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn
    210                 215                 220

Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn
225                 230                 235                 240

Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe
                245                 250                 255

Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp
            260                 265                 270
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```
Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp
        275                 280                 285

Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp
    290                 295                 300

Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser
305                 310                 315                 320

Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys
                325                 330                 335

Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe
            340                 345                 350

Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser
        355                 360                 365

Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp
    370                 375                 380

Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg
385                 390                 395                 400

Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu
                405                 410                 415

Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe
            420                 425                 430

Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile
        435                 440                 445

Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp
    450                 455                 460

Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu
465                 470                 475                 480

Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr
                485                 490                 495

Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser
            500                 505                 510

Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys
        515                 520                 525

Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln
    530                 535                 540

Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr
545                 550                 555                 560

Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp
                565                 570                 575

Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly
            580                 585                 590

Thr Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp
        595                 600                 605

Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr
    610                 615                 620

Leu Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala
625                 630                 635                 640

His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr
                645                 650                 655

Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp
            660                 665                 670

Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe
        675                 680                 685
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```
Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr Phe
    690                 695                 700

Lys Glu Asp Ile Gln Lys Ala Gln Val Ser Gly Gln Gly Asp Ser Leu
705                 710                 715                 720

His Glu His Ile Ala Asn Leu Ala Gly Ser Pro Ala Ile Lys Lys Gly
                725                 730                 735

Ile Leu Gln Thr Val Lys Val Val Asp Glu Leu Val Lys Val Met Gly
            740                 745                 750

Arg His Lys Pro Glu Asn Ile Val Ile Glu Met Ala Arg Glu Asn Gln
        755                 760                 765

Thr Thr Gln Lys Gly Gln Lys Asn Ser Arg Glu Arg Met Lys Arg Ile
    770                 775                 780

Glu Glu Gly Ile Lys Glu Leu Gly Ser Gln Ile Leu Lys Glu His Pro
785                 790                 795                 800

Val Glu Asn Thr Gln Leu Gln Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu
                805                 810                 815

Gln Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg
            820                 825                 830

Leu Ser Asp Tyr Asp Val Asp His Ile Val Pro Gln Ser Phe Leu Lys
        835                 840                 845

Asp Asp Ser Ile Asp Asn Lys Val Leu Thr Arg Ser Asp Lys Asn Arg
    850                 855                 860

Gly Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys
865                 870                 875                 880

Asn Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys
                885                 890                 895

Phe Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp
            900                 905                 910

Lys Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr
        915                 920                 925

Lys His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp
    930                 935                 940

Glu Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser
945                 950                 955                 960

Lys Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg
                965                 970                 975

Glu Ile Asn Asn Tyr His His Ala His Asp Ala Tyr Leu Asn Ala Val
            980                 985                 990

Val Gly Thr Ala Leu Ile Lys Lys  Tyr Pro Lys Leu Glu  Ser Glu Phe
        995                 1000                1005

Val Tyr  Gly Asp Tyr Lys Val  Tyr Asp Val Arg Lys  Met Ile Ala
    1010                1015                1020

Lys Ser  Glu Gln Glu Ile Gly  Lys Ala Thr Ala Lys  Tyr Phe Phe
    1025                1030                1035

Tyr Ser  Asn Ile Met Asn Phe  Phe Lys Thr Glu Ile  Thr Leu Ala
    1040                1045                1050

Asn Gly  Glu Ile Arg Lys Arg  Pro Leu Ile Glu Thr  Asn Gly Glu
    1055                1060                1065

Thr Gly  Glu Ile Val Trp Asp  Lys Gly Arg Asp Phe  Ala Thr Val
    1070                1075                1080

Arg Lys  Val Leu Ser Met Pro  Gln Val Asn Ile Val  Lys Lys Thr
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    1085                1090                1095

Glu Val  Gln Thr Gly Gly Phe  Ser Lys Glu Ser Ile  Leu Pro Lys
    1100                1105                1110

Arg Asn  Ser Asp Lys Leu Ile  Ala Arg Lys Lys Asp  Trp Asp Pro
    1115                1120                1125

Lys Lys  Tyr Gly Gly Phe Asp  Ser Pro Thr Val Ala  Tyr Ser Val
    1130                1135                1140

Leu Val  Val Ala Lys Val Glu  Lys Gly Lys Ser Lys  Lys Leu Lys
    1145                1150                1155

Ser Val  Lys Glu Leu Leu Gly  Ile Thr Ile Met Glu  Arg Ser Ser
    1160                1165                1170

Phe Glu  Lys Asn Pro Ile Asp  Phe Leu Glu Ala Lys  Gly Tyr Lys
    1175                1180                1185

Glu Val  Lys Lys Asp Leu Ile  Ile Lys Leu Pro Lys  Tyr Ser Leu
    1190                1195                1200

Phe Glu  Leu Glu Asn Gly Arg  Lys Arg Met Leu Ala  Ser Ala Gly
    1205                1210                1215

Glu Leu  Gln Lys Gly Asn Glu  Leu Ala Leu Pro Ser  Lys Tyr Val
    1220                1225                1230

Asn Phe  Leu Tyr Leu Ala Ser  His Tyr Glu Lys Leu  Lys Gly Ser
    1235                1240                1245

Pro Glu  Asp Asn Glu Gln Lys  Gln Leu Phe Val Glu  Gln His Lys
    1250                1255                1260

His Tyr  Leu Asp Glu Ile Ile  Glu Gln Ile Ser Glu  Phe Ser Lys
    1265                1270                1275

Arg Val  Ile Leu Ala Asp Ala  Asn Leu Asp Lys Val  Leu Ser Ala
    1280                1285                1290

Tyr Asn  Lys His Arg Asp Lys  Pro Ile Arg Glu Gln  Ala Glu Asn
    1295                1300                1305

Ile Ile  His Leu Phe Thr Leu  Thr Asn Leu Gly Ala  Pro Ala Ala
    1310                1315                1320

Phe Lys  Tyr Phe Asp Thr Thr  Ile Asp Arg Lys Arg  Tyr Thr Ser
    1325                1330                1335

Thr Lys  Glu Val Leu Asp Ala  Thr Leu Ile His Gln  Ser Ile Thr
    1340                1345                1350

Gly Leu  Tyr Glu Thr Arg Ile  Asp Leu Ser Gln Leu  Gly Gly Asp
    1355                1360                1365

Ala Ala  Ala Val Ser Lys Gly  Glu Glu Leu Phe Thr  Gly Val Val
    1370                1375                1380

Pro Ile  Leu Val Glu Leu Asp  Gly Asp Val Asn Gly  His Lys Phe
    1385                1390                1395

Ser Val  Ser Gly Glu Gly Glu  Gly Asp Ala Thr Tyr  Gly Lys Leu
    1400                1405                1410

Thr Leu  Lys Phe Ile Cys Thr  Thr Gly Lys Leu Pro  Val Pro Trp
    1415                1420                1425

Pro Thr  Leu Val Thr Thr Leu  Thr Tyr Gly Val Gln  Cys Phe Ser
    1430                1435                1440

Arg Tyr  Pro Asp His Met Lys  Gln His Asp Phe Phe  Lys Ser Ala
    1445                1450                1455

Met Pro  Glu Gly Tyr Val Gln  Glu Arg Thr Ile Phe  Phe Lys Asp
    1460                1465                1470
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Asp Gly  Asn Tyr Lys Thr Arg  Ala Glu Val Lys Phe  Glu Gly Asp
    1475                 1480                 1485

Thr Leu  Val Asn Arg Ile Glu  Leu Lys Gly Ile Asp  Phe Lys Glu
    1490                 1495                 1500

Asp Gly  Asn Ile Leu Gly His  Lys Leu Glu Tyr Asn  Tyr Asn Ser
    1505                 1510                 1515

His Asn  Val Tyr Ile Met Ala  Asp Lys Gln Lys Asn  Gly Ile Lys
    1520                 1525                 1530

Val Asn  Phe Lys Ile Arg His  Asn Ile Glu Asp Gly  Ser Val Gln
    1535                 1540                 1545

Leu Ala  Asp His Tyr Gln Gln  Asn Thr Pro Ile Gly  Asp Gly Pro
    1550                 1555                 1560

Val Leu  Leu Pro Asp Asn His  Tyr Leu Ser Thr Gln  Ser Ala Leu
    1565                 1570                 1575

Ser Lys  Asp Pro Asn Glu Lys  Arg Asp His Met Val  Leu Leu Glu
    1580                 1585                 1590

Phe Val  Thr Ala Ala Gly Ile  Thr Leu Gly Met Asp  Glu Leu Tyr
    1595                 1600                 1605

Lys Lys  Arg Pro Ala Ala Thr  Lys Lys Ala Gly Gln  Ala Lys Lys
    1610                 1615                 1620

Lys Lys
    1625


<210> SEQ ID NO 46
<211> LENGTH: 1664
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polypeptide

<400> SEQUENCE: 46

Met Asp Tyr Lys Asp His Asp Gly Asp Tyr Lys Asp His Asp Ile Asp
1               5                  10                  15

Tyr Lys Asp Asp Asp Asp Lys Met Ala Pro Lys Lys Lys Arg Lys Val
            20                  25                  30

Gly Ile His Gly Val Pro Ala Ala Asp Lys Lys Tyr Ser Ile Gly Leu
        35                  40                  45

Asp Ile Gly Thr Asn Ser Val Gly Trp Ala Val Ile Thr Asp Glu Tyr
    50                  55                  60

Lys Val Pro Ser Lys Lys Phe Lys Val Leu Gly Asn Thr Asp Arg His
65                  70                  75                  80

Ser Ile Lys Lys Asn Leu Ile Gly Ala Leu Leu Phe Asp Ser Gly Glu
                85                  90                  95

Thr Ala Glu Ala Thr Arg Leu Lys Arg Thr Ala Arg Arg Arg Tyr Thr
            100                 105                 110

Arg Arg Lys Asn Arg Ile Cys Tyr Leu Gln Glu Ile Phe Ser Asn Glu
        115                 120                 125

Met Ala Lys Val Asp Asp Ser Phe Phe His Arg Leu Glu Glu Ser Phe
    130                 135                 140

Leu Val Glu Glu Asp Lys Lys His Glu Arg His Pro Ile Phe Gly Asn
145                 150                 155                 160

Ile Val Asp Glu Val Ala Tyr His Glu Lys Tyr Pro Thr Ile Tyr His
                165                 170                 175
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Leu Arg Lys Lys Leu Val Asp Ser Thr Asp Lys Ala Asp Leu Arg Leu
            180                 185                 190

Ile Tyr Leu Ala Leu Ala His Met Ile Lys Phe Arg Gly His Phe Leu
        195                 200                 205

Ile Glu Gly Asp Leu Asn Pro Asp Asn Ser Asp Val Asp Lys Leu Phe
    210                 215                 220

Ile Gln Leu Val Gln Thr Tyr Asn Gln Leu Phe Glu Glu Asn Pro Ile
225                 230                 235                 240

Asn Ala Ser Gly Val Asp Ala Lys Ala Ile Leu Ser Ala Arg Leu Ser
                245                 250                 255

Lys Ser Arg Arg Leu Glu Asn Leu Ile Ala Gln Leu Pro Gly Glu Lys
            260                 265                 270

Lys Asn Gly Leu Phe Gly Asn Leu Ile Ala Leu Ser Leu Gly Leu Thr
        275                 280                 285

Pro Asn Phe Lys Ser Asn Phe Asp Leu Ala Glu Asp Ala Lys Leu Gln
    290                 295                 300

Leu Ser Lys Asp Thr Tyr Asp Asp Asp Leu Asp Asn Leu Leu Ala Gln
305                 310                 315                 320

Ile Gly Asp Gln Tyr Ala Asp Leu Phe Leu Ala Ala Lys Asn Leu Ser
                325                 330                 335

Asp Ala Ile Leu Leu Ser Asp Ile Leu Arg Val Asn Thr Glu Ile Thr
            340                 345                 350

Lys Ala Pro Leu Ser Ala Ser Met Ile Lys Arg Tyr Asp Glu His His
        355                 360                 365

Gln Asp Leu Thr Leu Leu Lys Ala Leu Val Arg Gln Gln Leu Pro Glu
    370                 375                 380

Lys Tyr Lys Glu Ile Phe Phe Asp Gln Ser Lys Asn Gly Tyr Ala Gly
385                 390                 395                 400

Tyr Ile Asp Gly Gly Ala Ser Gln Glu Glu Phe Tyr Lys Phe Ile Lys
                405                 410                 415

Pro Ile Leu Glu Lys Met Asp Gly Thr Glu Glu Leu Leu Val Lys Leu
            420                 425                 430

Asn Arg Glu Asp Leu Leu Arg Lys Gln Arg Thr Phe Asp Asn Gly Ser
        435                 440                 445

Ile Pro His Gln Ile His Leu Gly Glu Leu His Ala Ile Leu Arg Arg
    450                 455                 460

Gln Glu Asp Phe Tyr Pro Phe Leu Lys Asp Asn Arg Glu Lys Ile Glu
465                 470                 475                 480

Lys Ile Leu Thr Phe Arg Ile Pro Tyr Tyr Val Gly Pro Leu Ala Arg
                485                 490                 495

Gly Asn Ser Arg Phe Ala Trp Met Thr Arg Lys Ser Glu Glu Thr Ile
            500                 505                 510

Thr Pro Trp Asn Phe Glu Glu Val Val Asp Lys Gly Ala Ser Ala Gln
        515                 520                 525

Ser Phe Ile Glu Arg Met Thr Asn Phe Asp Lys Asn Leu Pro Asn Glu
    530                 535                 540

Lys Val Leu Pro Lys His Ser Leu Leu Tyr Glu Tyr Phe Thr Val Tyr
545                 550                 555                 560

Asn Glu Leu Thr Lys Val Lys Tyr Val Thr Glu Gly Met Arg Lys Pro
                565                 570                 575

Ala Phe Leu Ser Gly Glu Gln Lys Lys Ala Ile Val Asp Leu Leu Phe
            580                 585                 590
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Lys Thr Asn Arg Lys Val Thr Val Lys Gln Leu Lys Glu Asp Tyr Phe
        595                 600                 605

Lys Lys Ile Glu Cys Phe Asp Ser Val Glu Ile Ser Gly Val Glu Asp
    610                 615                 620

Arg Phe Asn Ala Ser Leu Gly Thr Tyr His Asp Leu Leu Lys Ile Ile
625                 630                 635                 640

Lys Asp Lys Asp Phe Leu Asp Asn Glu Glu Asn Glu Asp Ile Leu Glu
                645                 650                 655

Asp Ile Val Leu Thr Leu Thr Leu Phe Glu Asp Arg Glu Met Ile Glu
            660                 665                 670

Glu Arg Leu Lys Thr Tyr Ala His Leu Phe Asp Asp Lys Val Met Lys
        675                 680                 685

Gln Leu Lys Arg Arg Arg Tyr Thr Gly Trp Gly Arg Leu Ser Arg Lys
    690                 695                 700

Leu Ile Asn Gly Ile Arg Asp Lys Gln Ser Gly Lys Thr Ile Leu Asp
705                 710                 715                 720

Phe Leu Lys Ser Asp Gly Phe Ala Asn Arg Asn Phe Met Gln Leu Ile
                725                 730                 735

His Asp Asp Ser Leu Thr Phe Lys Glu Asp Ile Gln Lys Ala Gln Val
            740                 745                 750

Ser Gly Gln Gly Asp Ser Leu His Glu His Ile Ala Asn Leu Ala Gly
        755                 760                 765

Ser Pro Ala Ile Lys Lys Gly Ile Leu Gln Thr Val Lys Val Val Asp
    770                 775                 780

Glu Leu Val Lys Val Met Gly Arg His Lys Pro Glu Asn Ile Val Ile
785                 790                 795                 800

Glu Met Ala Arg Glu Asn Gln Thr Thr Gln Lys Gly Gln Lys Asn Ser
                805                 810                 815

Arg Glu Arg Met Lys Arg Ile Glu Glu Gly Ile Lys Glu Leu Gly Ser
            820                 825                 830

Gln Ile Leu Lys Glu His Pro Val Glu Asn Thr Gln Leu Gln Asn Glu
        835                 840                 845

Lys Leu Tyr Leu Tyr Tyr Leu Gln Asn Gly Arg Asp Met Tyr Val Asp
    850                 855                 860

Gln Glu Leu Asp Ile Asn Arg Leu Ser Asp Tyr Asp Val Asp His Ile
865                 870                 875                 880

Val Pro Gln Ser Phe Leu Lys Asp Asp Ser Ile Asp Asn Lys Val Leu
                885                 890                 895

Thr Arg Ser Asp Lys Asn Arg Gly Lys Ser Asp Asn Val Pro Ser Glu
            900                 905                 910

Glu Val Val Lys Lys Met Lys Asn Tyr Trp Arg Gln Leu Leu Asn Ala
        915                 920                 925

Lys Leu Ile Thr Gln Arg Lys Phe Asp Asn Leu Thr Lys Ala Glu Arg
    930                 935                 940

Gly Gly Leu Ser Glu Leu Asp Lys Ala Gly Phe Ile Lys Arg Gln Leu
945                 950                 955                 960

Val Glu Thr Arg Gln Ile Thr Lys His Val Ala Gln Ile Leu Asp Ser
                965                 970                 975

Arg Met Asn Thr Lys Tyr Asp Glu Asn Asp Lys Leu Ile Arg Glu Val
            980                 985                 990

Lys Val Ile Thr Leu Lys Ser Lys  Leu Val Ser Asp Phe  Arg Lys Asp
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        995                 1000                1005

Phe Gln  Phe Tyr Lys Val Arg  Glu Ile Asn Asn Tyr  His His Ala
    1010                1015                1020

His Asp  Ala Tyr Leu Asn Ala  Val Val Gly Thr Ala  Leu Ile Lys
    1025                1030                1035

Lys Tyr  Pro Lys Leu Glu Ser  Glu Phe Val Tyr Gly  Asp Tyr Lys
    1040                1045                1050

Val Tyr  Asp Val Arg Lys Met  Ile Ala Lys Ser Glu  Gln Glu Ile
    1055                1060                1065

Gly Lys  Ala Thr Ala Lys Tyr  Phe Phe Tyr Ser Asn  Ile Met Asn
    1070                1075                1080

Phe Phe  Lys Thr Glu Ile Thr  Leu Ala Asn Gly Glu  Ile Arg Lys
    1085                1090                1095

Arg Pro  Leu Ile Glu Thr Asn  Gly Glu Thr Gly Glu  Ile Val Trp
    1100                1105                1110

Asp Lys  Gly Arg Asp Phe Ala  Thr Val Arg Lys Val  Leu Ser Met
    1115                1120                1125

Pro Gln  Val Asn Ile Val Lys  Lys Thr Glu Val Gln  Thr Gly Gly
    1130                1135                1140

Phe Ser  Lys Glu Ser Ile Leu  Pro Lys Arg Asn Ser  Asp Lys Leu
    1145                1150                1155

Ile Ala  Arg Lys Lys Asp Trp  Asp Pro Lys Lys Tyr  Gly Gly Phe
    1160                1165                1170

Asp Ser  Pro Thr Val Ala Tyr  Ser Val Leu Val Val  Ala Lys Val
    1175                1180                1185

Glu Lys  Gly Lys Ser Lys Lys  Leu Lys Ser Val Lys  Glu Leu Leu
    1190                1195                1200

Gly Ile  Thr Ile Met Glu Arg  Ser Ser Phe Glu Lys  Asn Pro Ile
    1205                1210                1215

Asp Phe  Leu Glu Ala Lys Gly  Tyr Lys Glu Val Lys  Lys Asp Leu
    1220                1225                1230

Ile Ile  Lys Leu Pro Lys Tyr  Ser Leu Phe Glu Leu  Glu Asn Gly
    1235                1240                1245

Arg Lys  Arg Met Leu Ala Ser  Ala Gly Glu Leu Gln  Lys Gly Asn
    1250                1255                1260

Glu Leu  Ala Leu Pro Ser Lys  Tyr Val Asn Phe Leu  Tyr Leu Ala
    1265                1270                1275

Ser His  Tyr Glu Lys Leu Lys  Gly Ser Pro Glu Asp  Asn Glu Gln
    1280                1285                1290

Lys Gln  Leu Phe Val Glu Gln  His Lys His Tyr Leu  Asp Glu Ile
    1295                1300                1305

Ile Glu  Gln Ile Ser Glu Phe  Ser Lys Arg Val Ile  Leu Ala Asp
    1310                1315                1320

Ala Asn  Leu Asp Lys Val Leu  Ser Ala Tyr Asn Lys  His Arg Asp
    1325                1330                1335

Lys Pro  Ile Arg Glu Gln Ala  Glu Asn Ile Ile His  Leu Phe Thr
    1340                1345                1350

Leu Thr  Asn Leu Gly Ala Pro  Ala Ala Phe Lys Tyr  Phe Asp Thr
    1355                1360                1365

Thr Ile  Asp Arg Lys Arg Tyr  Thr Ser Thr Lys Glu  Val Leu Asp
    1370                1375                1380
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Ala Thr  Leu Ile His Gln Ser  Ile Thr Gly Leu Tyr  Glu Thr Arg
    1385                1390                1395

Ile Asp  Leu Ser Gln Leu Gly  Gly Asp Ala Ala Ala  Val Ser Lys
    1400                1405                1410

Gly Glu  Glu Leu Phe Thr Gly  Val Val Pro Ile Leu  Val Glu Leu
    1415                1420                1425

Asp Gly  Asp Val Asn Gly His  Lys Phe Ser Val Ser  Gly Glu Gly
    1430                1435                1440

Glu Gly  Asp Ala Thr Tyr Gly  Lys Leu Thr Leu Lys  Phe Ile Cys
    1445                1450                1455

Thr Thr  Gly Lys Leu Pro Val  Pro Trp Pro Thr Leu  Val Thr Thr
    1460                1465                1470

Leu Thr  Tyr Gly Val Gln Cys  Phe Ser Arg Tyr Pro  Asp His Met
    1475                1480                1485

Lys Gln  His Asp Phe Phe Lys  Ser Ala Met Pro Glu  Gly Tyr Val
    1490                1495                1500

Gln Glu  Arg Thr Ile Phe Phe  Lys Asp Asp Gly Asn  Tyr Lys Thr
    1505                1510                1515

Arg Ala  Glu Val Lys Phe Glu  Gly Asp Thr Leu Val  Asn Arg Ile
    1520                1525                1530

Glu Leu  Lys Gly Ile Asp Phe  Lys Glu Asp Gly Asn  Ile Leu Gly
    1535                1540                1545

His Lys  Leu Glu Tyr Asn Tyr  Asn Ser His Asn Val  Tyr Ile Met
    1550                1555                1560

Ala Asp  Lys Gln Lys Asn Gly  Ile Lys Val Asn Phe  Lys Ile Arg
    1565                1570                1575

His Asn  Ile Glu Asp Gly Ser  Val Gln Leu Ala Asp  His Tyr Gln
    1580                1585                1590

Gln Asn  Thr Pro Ile Gly Asp  Gly Pro Val Leu Leu  Pro Asp Asn
    1595                1600                1605

His Tyr  Leu Ser Thr Gln Ser  Ala Leu Ser Lys Asp  Pro Asn Glu
    1610                1615                1620

Lys Arg  Asp His Met Val Leu  Leu Glu Phe Val Thr  Ala Ala Gly
    1625                1630                1635

Ile Thr  Leu Gly Met Asp Glu  Leu Tyr Lys Lys Arg  Pro Ala Ala
    1640                1645                1650

Thr Lys  Lys Ala Gly Gln Ala  Lys Lys Lys Lys
    1655                1660


<210> SEQ ID NO 47
<211> LENGTH: 1423
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polypeptide

<400> SEQUENCE: 47

Met Asp Tyr Lys Asp His Asp Gly Asp Tyr Lys Asp His Asp Ile Asp
1               5                  10                  15

Tyr Lys Asp Asp Asp Asp Lys Met Ala Pro Lys Lys Lys Arg Lys Val
            20                  25                  30

Gly Ile His Gly Val Pro Ala Ala Asp Lys Lys Tyr Ser Ile Gly Leu
        35                  40                  45
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Asp Ile Gly Thr Asn Ser Val Gly Trp Ala Val Ile Thr Asp Glu Tyr
    50                  55                  60

Lys Val Pro Ser Lys Lys Phe Lys Val Leu Gly Asn Thr Asp Arg His
65                  70                  75                  80

Ser Ile Lys Lys Asn Leu Ile Gly Ala Leu Leu Phe Asp Ser Gly Glu
                85                  90                  95

Thr Ala Glu Ala Thr Arg Leu Lys Arg Thr Ala Arg Arg Arg Tyr Thr
            100                 105                 110

Arg Arg Lys Asn Arg Ile Cys Tyr Leu Gln Glu Ile Phe Ser Asn Glu
        115                 120                 125

Met Ala Lys Val Asp Asp Ser Phe Phe His Arg Leu Glu Glu Ser Phe
    130                 135                 140

Leu Val Glu Glu Asp Lys Lys His Glu Arg His Pro Ile Phe Gly Asn
145                 150                 155                 160

Ile Val Asp Glu Val Ala Tyr His Glu Lys Tyr Pro Thr Ile Tyr His
                165                 170                 175

Leu Arg Lys Lys Leu Val Asp Ser Thr Asp Lys Ala Asp Leu Arg Leu
            180                 185                 190

Ile Tyr Leu Ala Leu Ala His Met Ile Lys Phe Arg Gly His Phe Leu
        195                 200                 205

Ile Glu Gly Asp Leu Asn Pro Asp Asn Ser Asp Val Asp Lys Leu Phe
    210                 215                 220

Ile Gln Leu Val Gln Thr Tyr Asn Gln Leu Phe Glu Glu Asn Pro Ile
225                 230                 235                 240

Asn Ala Ser Gly Val Asp Ala Lys Ala Ile Leu Ser Ala Arg Leu Ser
                245                 250                 255

Lys Ser Arg Arg Leu Glu Asn Leu Ile Ala Gln Leu Pro Gly Glu Lys
            260                 265                 270

Lys Asn Gly Leu Phe Gly Asn Leu Ile Ala Leu Ser Leu Gly Leu Thr
        275                 280                 285

Pro Asn Phe Lys Ser Asn Phe Asp Leu Ala Glu Asp Ala Lys Leu Gln
    290                 295                 300

Leu Ser Lys Asp Thr Tyr Asp Asp Asp Leu Asp Asn Leu Leu Ala Gln
305                 310                 315                 320

Ile Gly Asp Gln Tyr Ala Asp Leu Phe Leu Ala Ala Lys Asn Leu Ser
                325                 330                 335

Asp Ala Ile Leu Leu Ser Asp Ile Leu Arg Val Asn Thr Glu Ile Thr
            340                 345                 350

Lys Ala Pro Leu Ser Ala Ser Met Ile Lys Arg Tyr Asp Glu His His
        355                 360                 365

Gln Asp Leu Thr Leu Leu Lys Ala Leu Val Arg Gln Gln Leu Pro Glu
    370                 375                 380

Lys Tyr Lys Glu Ile Phe Phe Asp Gln Ser Lys Asn Gly Tyr Ala Gly
385                 390                 395                 400

Tyr Ile Asp Gly Gly Ala Ser Gln Glu Glu Phe Tyr Lys Phe Ile Lys
                405                 410                 415

Pro Ile Leu Glu Lys Met Asp Gly Thr Glu Glu Leu Leu Val Lys Leu
            420                 425                 430

Asn Arg Glu Asp Leu Leu Arg Lys Gln Arg Thr Phe Asp Asn Gly Ser
        435                 440                 445

Ile Pro His Gln Ile His Leu Gly Glu Leu His Ala Ile Leu Arg Arg
    450                 455                 460
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```
Gln Glu Asp Phe Tyr Pro Phe Leu Lys Asp Asn Arg Glu Lys Ile Glu
465                 470                 475                 480

Lys Ile Leu Thr Phe Arg Ile Pro Tyr Tyr Val Gly Pro Leu Ala Arg
                485                 490                 495

Gly Asn Ser Arg Phe Ala Trp Met Thr Arg Lys Ser Glu Glu Thr Ile
            500                 505                 510

Thr Pro Trp Asn Phe Glu Glu Val Val Asp Lys Gly Ala Ser Ala Gln
        515                 520                 525

Ser Phe Ile Glu Arg Met Thr Asn Phe Asp Lys Asn Leu Pro Asn Glu
    530                 535                 540

Lys Val Leu Pro Lys His Ser Leu Leu Tyr Glu Tyr Phe Thr Val Tyr
545                 550                 555                 560

Asn Glu Leu Thr Lys Val Lys Tyr Val Thr Glu Gly Met Arg Lys Pro
                565                 570                 575

Ala Phe Leu Ser Gly Glu Gln Lys Lys Ala Ile Val Asp Leu Leu Phe
            580                 585                 590

Lys Thr Asn Arg Lys Val Thr Val Lys Gln Leu Lys Glu Asp Tyr Phe
        595                 600                 605

Lys Lys Ile Glu Cys Phe Asp Ser Val Glu Ile Ser Gly Val Glu Asp
    610                 615                 620

Arg Phe Asn Ala Ser Leu Gly Thr Tyr His Asp Leu Leu Lys Ile Ile
625                 630                 635                 640

Lys Asp Lys Asp Phe Leu Asp Asn Glu Glu Asn Glu Asp Ile Leu Glu
                645                 650                 655

Asp Ile Val Leu Thr Leu Thr Leu Phe Glu Asp Arg Glu Met Ile Glu
            660                 665                 670

Glu Arg Leu Lys Thr Tyr Ala His Leu Phe Asp Asp Lys Val Met Lys
        675                 680                 685

Gln Leu Lys Arg Arg Arg Tyr Thr Gly Trp Gly Arg Leu Ser Arg Lys
    690                 695                 700

Leu Ile Asn Gly Ile Arg Asp Lys Gln Ser Gly Lys Thr Ile Leu Asp
705                 710                 715                 720

Phe Leu Lys Ser Asp Gly Phe Ala Asn Arg Asn Phe Met Gln Leu Ile
                725                 730                 735

His Asp Asp Ser Leu Thr Phe Lys Glu Asp Ile Gln Lys Ala Gln Val
            740                 745                 750

Ser Gly Gln Gly Asp Ser Leu His Glu His Ile Ala Asn Leu Ala Gly
        755                 760                 765

Ser Pro Ala Ile Lys Lys Gly Ile Leu Gln Thr Val Lys Val Val Asp
    770                 775                 780

Glu Leu Val Lys Val Met Gly Arg His Lys Pro Glu Asn Ile Val Ile
785                 790                 795                 800

Glu Met Ala Arg Glu Asn Gln Thr Thr Gln Lys Gly Gln Lys Asn Ser
                805                 810                 815

Arg Glu Arg Met Lys Arg Ile Glu Glu Gly Ile Lys Glu Leu Gly Ser
            820                 825                 830

Gln Ile Leu Lys Glu His Pro Val Glu Asn Thr Gln Leu Gln Asn Glu
        835                 840                 845

Lys Leu Tyr Leu Tyr Tyr Leu Gln Asn Gly Arg Asp Met Tyr Val Asp
    850                 855                 860

Gln Glu Leu Asp Ile Asn Arg Leu Ser Asp Tyr Asp Val Asp His Ile
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865                 870                 875                 880

Val Pro Gln Ser Phe Leu Lys Asp Asp Ser Ile Asp Asn Lys Val Leu
                885                 890                 895

Thr Arg Ser Asp Lys Asn Arg Gly Lys Ser Asp Asn Val Pro Ser Glu
            900                 905                 910

Glu Val Val Lys Lys Met Lys Asn Tyr Trp Arg Gln Leu Leu Asn Ala
        915                 920                 925

Lys Leu Ile Thr Gln Arg Lys Phe Asp Asn Leu Thr Lys Ala Glu Arg
    930                 935                 940

Gly Gly Leu Ser Glu Leu Asp Lys Ala Gly Phe Ile Lys Arg Gln Leu
945                 950                 955                 960

Val Glu Thr Arg Gln Ile Thr Lys His Val Ala Gln Ile Leu Asp Ser
                965                 970                 975

Arg Met Asn Thr Lys Tyr Asp Glu Asn Asp Lys Leu Ile Arg Glu Val
            980                 985                 990

Lys Val Ile Thr Leu Lys Ser Lys  Leu Val Ser Asp Phe  Arg Lys Asp
        995                 1000                1005

Phe Gln  Phe Tyr Lys Val Arg  Glu Ile Asn Asn Tyr  His His Ala
    1010                1015                1020

His Asp  Ala Tyr Leu Asn Ala  Val Val Gly Thr Ala  Leu Ile Lys
    1025                1030                1035

Lys Tyr  Pro Lys Leu Glu Ser  Glu Phe Val Tyr Gly  Asp Tyr Lys
    1040                1045                1050

Val Tyr  Asp Val Arg Lys Met  Ile Ala Lys Ser Glu  Gln Glu Ile
    1055                1060                1065

Gly Lys  Ala Thr Ala Lys Tyr  Phe Phe Tyr Ser Asn  Ile Met Asn
    1070                1075                1080

Phe Phe  Lys Thr Glu Ile Thr  Leu Ala Asn Gly Glu  Ile Arg Lys
    1085                1090                1095

Arg Pro  Leu Ile Glu Thr Asn  Gly Glu Thr Gly Glu  Ile Val Trp
    1100                1105                1110

Asp Lys  Gly Arg Asp Phe Ala  Thr Val Arg Lys Val  Leu Ser Met
    1115                1120                1125

Pro Gln  Val Asn Ile Val Lys  Lys Thr Glu Val Gln  Thr Gly Gly
    1130                1135                1140

Phe Ser  Lys Glu Ser Ile Leu  Pro Lys Arg Asn Ser  Asp Lys Leu
    1145                1150                1155

Ile Ala  Arg Lys Lys Asp Trp  Asp Pro Lys Lys Tyr  Gly Gly Phe
    1160                1165                1170

Asp Ser  Pro Thr Val Ala Tyr  Ser Val Leu Val Val  Ala Lys Val
    1175                1180                1185

Glu Lys  Gly Lys Ser Lys Lys  Leu Lys Ser Val Lys  Glu Leu Leu
    1190                1195                1200

Gly Ile  Thr Ile Met Glu Arg  Ser Ser Phe Glu Lys  Asn Pro Ile
    1205                1210                1215

Asp Phe  Leu Glu Ala Lys Gly  Tyr Lys Glu Val Lys  Lys Asp Leu
    1220                1225                1230

Ile Ile  Lys Leu Pro Lys Tyr  Ser Leu Phe Glu Leu  Glu Asn Gly
    1235                1240                1245

Arg Lys  Arg Met Leu Ala Ser  Ala Gly Glu Leu Gln  Lys Gly Asn
    1250                1255                1260
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Glu Leu  Ala Leu Pro Ser Lys  Tyr Val Asn Phe Leu  Tyr Leu Ala
    1265                1270                1275

Ser His  Tyr Glu Lys Leu Lys  Gly Ser Pro Glu Asp  Asn Glu Gln
    1280                1285                1290

Lys Gln  Leu Phe Val Glu Gln  His Lys His Tyr Leu  Asp Glu Ile
    1295                1300                1305

Ile Glu  Gln Ile Ser Glu Phe  Ser Lys Arg Val Ile  Leu Ala Asp
    1310                1315                1320

Ala Asn  Leu Asp Lys Val Leu  Ser Ala Tyr Asn Lys  His Arg Asp
    1325                1330                1335

Lys Pro  Ile Arg Glu Gln Ala  Glu Asn Ile Ile His  Leu Phe Thr
    1340                1345                1350

Leu Thr  Asn Leu Gly Ala Pro  Ala Ala Phe Lys Tyr  Phe Asp Thr
    1355                1360                1365

Thr Ile  Asp Arg Lys Arg Tyr  Thr Ser Thr Lys Glu  Val Leu Asp
    1370                1375                1380

Ala Thr  Leu Ile His Gln Ser  Ile Thr Gly Leu Tyr  Glu Thr Arg
    1385                1390                1395

Ile Asp  Leu Ser Gln Leu Gly  Gly Asp Lys Arg Pro  Ala Ala Thr
    1400                1405                1410

Lys Lys  Ala Gly Gln Ala Lys  Lys Lys Lys
    1415                1420


<210> SEQ ID NO 48
<211> LENGTH: 483
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polypeptide

<400> SEQUENCE: 48

Met Phe Leu Phe Leu Ser Leu Thr Ser Phe Leu Ser Ser Ser Arg Thr
1               5                   10                  15

Leu Val Ser Lys Gly Glu Glu Asp Asn Met Ala Ile Ile Lys Glu Phe
            20                  25                  30

Met Arg Phe Lys Val His Met Glu Gly Ser Val Asn Gly His Glu Phe
        35                  40                  45

Glu Ile Glu Gly Glu Gly Glu Gly Arg Pro Tyr Glu Gly Thr Gln Thr
    50                  55                  60

Ala Lys Leu Lys Val Thr Lys Gly Gly Pro Leu Pro Phe Ala Trp Asp
65                  70                  75                  80

Ile Leu Ser Pro Gln Phe Met Tyr Gly Ser Lys Ala Tyr Val Lys His
                85                  90                  95

Pro Ala Asp Ile Pro Asp Tyr Leu Lys Leu Ser Phe Pro Glu Gly Phe
            100                 105                 110

Lys Trp Glu Arg Val Met Asn Phe Glu Asp Gly Gly Val Val Thr Val
        115                 120                 125

Thr Gln Asp Ser Ser Leu Gln Asp Gly Glu Phe Ile Tyr Lys Val Lys
    130                 135                 140

Leu Arg Gly Thr Asn Phe Pro Ser Asp Gly Pro Val Met Gln Lys Lys
145                 150                 155                 160

Thr Met Gly Trp Glu Ala Ser Ser Glu Arg Met Tyr Pro Glu Asp Gly
                165                 170                 175
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Ala Leu Lys Gly Glu Ile Lys Gln Arg Leu Lys Leu Lys Asp Gly Gly
            180                 185                 190

His Tyr Asp Ala Glu Val Lys Thr Thr Tyr Lys Ala Lys Lys Pro Val
        195                 200                 205

Gln Leu Pro Gly Ala Tyr Asn Val Asn Ile Lys Leu Asp Ile Thr Ser
    210                 215                 220

His Asn Glu Asp Tyr Thr Ile Val Glu Gln Tyr Glu Arg Ala Glu Gly
225                 230                 235                 240

Arg His Ser Thr Gly Gly Met Asp Glu Leu Tyr Lys Gly Ser Lys Gln
                245                 250                 255

Leu Glu Glu Leu Leu Ser Thr Ser Phe Asp Ile Gln Phe Asn Asp Leu
            260                 265                 270

Thr Leu Leu Glu Thr Ala Phe Thr His Thr Ser Tyr Ala Asn Glu His
        275                 280                 285

Arg Leu Leu Asn Val Ser His Asn Glu Arg Leu Glu Phe Leu Gly Asp
    290                 295                 300

Ala Val Leu Gln Leu Ile Ile Ser Glu Tyr Leu Phe Ala Lys Tyr Pro
305                 310                 315                 320

Lys Lys Thr Glu Gly Asp Met Ser Lys Leu Arg Ser Met Ile Val Arg
                325                 330                 335

Glu Glu Ser Leu Ala Gly Phe Ser Arg Phe Cys Ser Phe Asp Ala Tyr
            340                 345                 350

Ile Lys Leu Gly Lys Gly Glu Glu Lys Ser Gly Gly Arg Arg Arg Asp
        355                 360                 365

Thr Ile Leu Gly Asp Leu Phe Glu Ala Phe Leu Gly Ala Leu Leu Leu
    370                 375                 380

Asp Lys Gly Ile Asp Ala Val Arg Arg Phe Leu Lys Gln Val Met Ile
385                 390                 395                 400

Pro Gln Val Glu Lys Gly Asn Phe Glu Arg Val Lys Asp Tyr Lys Thr
                405                 410                 415

Cys Leu Gln Glu Phe Leu Gln Thr Lys Gly Asp Val Ala Ile Asp Tyr
            420                 425                 430

Gln Val Ile Ser Glu Lys Gly Pro Ala His Ala Lys Gln Phe Glu Val
        435                 440                 445

Ser Ile Val Val Asn Gly Ala Val Leu Ser Lys Gly Leu Gly Lys Ser
    450                 455                 460

Lys Lys Leu Ala Glu Gln Asp Ala Ala Lys Asn Ala Leu Ala Gln Leu
465                 470                 475                 480

Ser Glu Val


<210> SEQ ID NO 49
<211> LENGTH: 483
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polypeptide

<400> SEQUENCE: 49

Met Lys Gln Leu Glu Glu Leu Leu Ser Thr Ser Phe Asp Ile Gln Phe
1               5                   10                  15

Asn Asp Leu Thr Leu Leu Glu Thr Ala Phe Thr His Thr Ser Tyr Ala
            20                  25                  30

Asn Glu His Arg Leu Leu Asn Val Ser His Asn Glu Arg Leu Glu Phe
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        35                  40                  45

Leu Gly Asp Ala Val Leu Gln Leu Ile Ile Ser Glu Tyr Leu Phe Ala
    50                  55                  60

Lys Tyr Pro Lys Lys Thr Glu Gly Asp Met Ser Lys Leu Arg Ser Met
65                  70                  75                  80

Ile Val Arg Glu Glu Ser Leu Ala Gly Phe Ser Arg Phe Cys Ser Phe
                85                  90                  95

Asp Ala Tyr Ile Lys Leu Gly Lys Gly Glu Glu Lys Ser Gly Gly Arg
            100                 105                 110

Arg Arg Asp Thr Ile Leu Gly Asp Leu Phe Glu Ala Phe Leu Gly Ala
        115                 120                 125

Leu Leu Leu Asp Lys Gly Ile Asp Ala Val Arg Arg Phe Leu Lys Gln
    130                 135                 140

Val Met Ile Pro Gln Val Glu Lys Gly Asn Phe Glu Arg Val Lys Asp
145                 150                 155                 160

Tyr Lys Thr Cys Leu Gln Glu Phe Leu Gln Thr Lys Gly Asp Val Ala
                165                 170                 175

Ile Asp Tyr Gln Val Ile Ser Glu Lys Gly Pro Ala His Ala Lys Gln
            180                 185                 190

Phe Glu Val Ser Ile Val Val Asn Gly Ala Val Leu Ser Lys Gly Leu
        195                 200                 205

Gly Lys Ser Lys Lys Leu Ala Glu Gln Asp Ala Ala Lys Asn Ala Leu
    210                 215                 220

Ala Gln Leu Ser Glu Val Gly Ser Val Ser Lys Gly Glu Glu Asp Asn
225                 230                 235                 240

Met Ala Ile Ile Lys Glu Phe Met Arg Phe Lys Val His Met Glu Gly
                245                 250                 255

Ser Val Asn Gly His Glu Phe Glu Ile Glu Gly Glu Gly Glu Gly Arg
            260                 265                 270

Pro Tyr Glu Gly Thr Gln Thr Ala Lys Leu Lys Val Thr Lys Gly Gly
        275                 280                 285

Pro Leu Pro Phe Ala Trp Asp Ile Leu Ser Pro Gln Phe Met Tyr Gly
    290                 295                 300

Ser Lys Ala Tyr Val Lys His Pro Ala Asp Ile Pro Asp Tyr Leu Lys
305                 310                 315                 320

Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val Met Asn Phe Glu
                325                 330                 335

Asp Gly Gly Val Val Thr Val Thr Gln Asp Ser Ser Leu Gln Asp Gly
            340                 345                 350

Glu Phe Ile Tyr Lys Val Lys Leu Arg Gly Thr Asn Phe Pro Ser Asp
        355                 360                 365

Gly Pro Val Met Gln Lys Lys Thr Met Gly Trp Glu Ala Ser Ser Glu
    370                 375                 380

Arg Met Tyr Pro Glu Asp Gly Ala Leu Lys Gly Glu Ile Lys Gln Arg
385                 390                 395                 400

Leu Lys Leu Lys Asp Gly Gly His Tyr Asp Ala Glu Val Lys Thr Thr
                405                 410                 415

Tyr Lys Ala Lys Lys Pro Val Gln Leu Pro Gly Ala Tyr Asn Val Asn
            420                 425                 430

Ile Lys Leu Asp Ile Thr Ser His Asn Glu Asp Tyr Thr Ile Val Glu
        435                 440                 445
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```
Gln Tyr Glu Arg Ala Glu Gly Arg His Ser Thr Gly Gly Met Asp Glu
    450                 455                 460

Leu Tyr Lys Lys Arg Pro Ala Ala Thr Lys Lys Ala Gly Gln Ala Lys
465                 470                 475                 480

Lys Lys Lys


<210> SEQ ID NO 50
<211> LENGTH: 1423
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polypeptide

<400> SEQUENCE: 50

Met Asp Tyr Lys Asp His Asp Gly Asp Tyr Lys Asp His Asp Ile Asp
1               5                   10                  15

Tyr Lys Asp Asp Asp Asp Lys Met Ala Pro Lys Lys Lys Arg Lys Val
            20                  25                  30

Gly Ile His Gly Val Pro Ala Ala Asp Lys Lys Tyr Ser Ile Gly Leu
        35                  40                  45

Ala Ile Gly Thr Asn Ser Val Gly Trp Ala Val Ile Thr Asp Glu Tyr
    50                  55                  60

Lys Val Pro Ser Lys Lys Phe Lys Val Leu Gly Asn Thr Asp Arg His
65                  70                  75                  80

Ser Ile Lys Lys Asn Leu Ile Gly Ala Leu Leu Phe Asp Ser Gly Glu
                85                  90                  95

Thr Ala Glu Ala Thr Arg Leu Lys Arg Thr Ala Arg Arg Arg Tyr Thr
            100                 105                 110

Arg Arg Lys Asn Arg Ile Cys Tyr Leu Gln Glu Ile Phe Ser Asn Glu
        115                 120                 125

Met Ala Lys Val Asp Asp Ser Phe Phe His Arg Leu Glu Glu Ser Phe
    130                 135                 140

Leu Val Glu Glu Asp Lys Lys His Glu Arg His Pro Ile Phe Gly Asn
145                 150                 155                 160

Ile Val Asp Glu Val Ala Tyr His Glu Lys Tyr Pro Thr Ile Tyr His
                165                 170                 175

Leu Arg Lys Lys Leu Val Asp Ser Thr Asp Lys Ala Asp Leu Arg Leu
            180                 185                 190

Ile Tyr Leu Ala Leu Ala His Met Ile Lys Phe Arg Gly His Phe Leu
        195                 200                 205

Ile Glu Gly Asp Leu Asn Pro Asp Asn Ser Asp Val Asp Lys Leu Phe
    210                 215                 220

Ile Gln Leu Val Gln Thr Tyr Asn Gln Leu Phe Glu Glu Asn Pro Ile
225                 230                 235                 240

Asn Ala Ser Gly Val Asp Ala Lys Ala Ile Leu Ser Ala Arg Leu Ser
                245                 250                 255

Lys Ser Arg Arg Leu Glu Asn Leu Ile Ala Gln Leu Pro Gly Glu Lys
            260                 265                 270

Lys Asn Gly Leu Phe Gly Asn Leu Ile Ala Leu Ser Leu Gly Leu Thr
        275                 280                 285

Pro Asn Phe Lys Ser Asn Phe Asp Leu Ala Glu Asp Ala Lys Leu Gln
    290                 295                 300

Leu Ser Lys Asp Thr Tyr Asp Asp Asp Leu Asp Asn Leu Leu Ala Gln
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305                 310                 315                 320

Ile Gly Asp Gln Tyr Ala Asp Leu Phe Leu Ala Ala Lys Asn Leu Ser
                325                 330                 335

Asp Ala Ile Leu Leu Ser Asp Ile Leu Arg Val Asn Thr Glu Ile Thr
            340                 345                 350

Lys Ala Pro Leu Ser Ala Ser Met Ile Lys Arg Tyr Asp Glu His His
        355                 360                 365

Gln Asp Leu Thr Leu Leu Lys Ala Leu Val Arg Gln Gln Leu Pro Glu
    370                 375                 380

Lys Tyr Lys Glu Ile Phe Phe Asp Gln Ser Lys Asn Gly Tyr Ala Gly
385                 390                 395                 400

Tyr Ile Asp Gly Gly Ala Ser Gln Glu Glu Phe Tyr Lys Phe Ile Lys
                405                 410                 415

Pro Ile Leu Glu Lys Met Asp Gly Thr Glu Glu Leu Leu Val Lys Leu
            420                 425                 430

Asn Arg Glu Asp Leu Leu Arg Lys Gln Arg Thr Phe Asp Asn Gly Ser
        435                 440                 445

Ile Pro His Gln Ile His Leu Gly Glu Leu His Ala Ile Leu Arg Arg
    450                 455                 460

Gln Glu Asp Phe Tyr Pro Phe Leu Lys Asp Asn Arg Glu Lys Ile Glu
465                 470                 475                 480

Lys Ile Leu Thr Phe Arg Ile Pro Tyr Tyr Val Gly Pro Leu Ala Arg
                485                 490                 495

Gly Asn Ser Arg Phe Ala Trp Met Thr Arg Lys Ser Glu Glu Thr Ile
            500                 505                 510

Thr Pro Trp Asn Phe Glu Glu Val Val Asp Lys Gly Ala Ser Ala Gln
        515                 520                 525

Ser Phe Ile Glu Arg Met Thr Asn Phe Asp Lys Asn Leu Pro Asn Glu
    530                 535                 540

Lys Val Leu Pro Lys His Ser Leu Leu Tyr Glu Tyr Phe Thr Val Tyr
545                 550                 555                 560

Asn Glu Leu Thr Lys Val Lys Tyr Val Thr Glu Gly Met Arg Lys Pro
                565                 570                 575

Ala Phe Leu Ser Gly Glu Gln Lys Lys Ala Ile Val Asp Leu Leu Phe
            580                 585                 590

Lys Thr Asn Arg Lys Val Thr Val Lys Gln Leu Lys Glu Asp Tyr Phe
        595                 600                 605

Lys Lys Ile Glu Cys Phe Asp Ser Val Glu Ile Ser Gly Val Glu Asp
    610                 615                 620

Arg Phe Asn Ala Ser Leu Gly Thr Tyr His Asp Leu Leu Lys Ile Ile
625                 630                 635                 640

Lys Asp Lys Asp Phe Leu Asp Asn Glu Glu Asn Glu Asp Ile Leu Glu
                645                 650                 655

Asp Ile Val Leu Thr Leu Thr Leu Phe Glu Asp Arg Glu Met Ile Glu
            660                 665                 670

Glu Arg Leu Lys Thr Tyr Ala His Leu Phe Asp Asp Lys Val Met Lys
        675                 680                 685

Gln Leu Lys Arg Arg Arg Tyr Thr Gly Trp Gly Arg Leu Ser Arg Lys
    690                 695                 700

Leu Ile Asn Gly Ile Arg Asp Lys Gln Ser Gly Lys Thr Ile Leu Asp
705                 710                 715                 720
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```
Phe Leu Lys Ser Asp Gly Phe Ala Asn Arg Asn Phe Met Gln Leu Ile
                725                 730                 735

His Asp Asp Ser Leu Thr Phe Lys Glu Asp Ile Gln Lys Ala Gln Val
            740                 745                 750

Ser Gly Gln Gly Asp Ser Leu His Glu His Ile Ala Asn Leu Ala Gly
        755                 760                 765

Ser Pro Ala Ile Lys Lys Gly Ile Leu Gln Thr Val Lys Val Val Asp
    770                 775                 780

Glu Leu Val Lys Val Met Gly Arg His Lys Pro Glu Asn Ile Val Ile
785                 790                 795                 800

Glu Met Ala Arg Glu Asn Gln Thr Thr Gln Lys Gly Gln Lys Asn Ser
                805                 810                 815

Arg Glu Arg Met Lys Arg Ile Glu Glu Gly Ile Lys Glu Leu Gly Ser
            820                 825                 830

Gln Ile Leu Lys Glu His Pro Val Glu Asn Thr Gln Leu Gln Asn Glu
        835                 840                 845

Lys Leu Tyr Leu Tyr Tyr Leu Gln Asn Gly Arg Asp Met Tyr Val Asp
    850                 855                 860

Gln Glu Leu Asp Ile Asn Arg Leu Ser Asp Tyr Asp Val Asp His Ile
865                 870                 875                 880

Val Pro Gln Ser Phe Leu Lys Asp Asp Ser Ile Asp Asn Lys Val Leu
                885                 890                 895

Thr Arg Ser Asp Lys Asn Arg Gly Lys Ser Asp Asn Val Pro Ser Glu
            900                 905                 910

Glu Val Val Lys Lys Met Lys Asn Tyr Trp Arg Gln Leu Leu Asn Ala
        915                 920                 925

Lys Leu Ile Thr Gln Arg Lys Phe Asp Asn Leu Thr Lys Ala Glu Arg
    930                 935                 940

Gly Gly Leu Ser Glu Leu Asp Lys Ala Gly Phe Ile Lys Arg Gln Leu
945                 950                 955                 960

Val Glu Thr Arg Gln Ile Thr Lys His Val Ala Gln Ile Leu Asp Ser
                965                 970                 975

Arg Met Asn Thr Lys Tyr Asp Glu Asn Asp Lys Leu Ile Arg Glu Val
            980                 985                 990

Lys Val Ile Thr Leu Lys Ser Lys  Leu Val Ser Asp Phe  Arg Lys Asp
        995                 1000                1005

Phe Gln  Phe Tyr Lys Val Arg  Glu Ile Asn Asn Tyr  His His Ala
    1010                1015                1020

His Asp  Ala Tyr Leu Asn Ala  Val Val Gly Thr Ala  Leu Ile Lys
    1025                1030                1035

Lys Tyr  Pro Lys Leu Glu Ser  Glu Phe Val Tyr Gly  Asp Tyr Lys
    1040                1045                1050

Val Tyr  Asp Val Arg Lys Met  Ile Ala Lys Ser Glu  Gln Glu Ile
    1055                1060                1065

Gly Lys  Ala Thr Ala Lys Tyr  Phe Phe Tyr Ser Asn  Ile Met Asn
    1070                1075                1080

Phe Phe  Lys Thr Glu Ile Thr  Leu Ala Asn Gly Glu  Ile Arg Lys
    1085                1090                1095

Arg Pro  Leu Ile Glu Thr Asn  Gly Glu Thr Gly Glu  Ile Val Trp
    1100                1105                1110

Asp Lys  Gly Arg Asp Phe Ala  Thr Val Arg Lys Val  Leu Ser Met
    1115                1120                1125
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```
Pro Gln  Val Asn Ile Val Lys  Lys Thr Glu Val Gln  Thr Gly Gly
    1130                1135                1140

Phe Ser  Lys Glu Ser Ile Leu  Pro Lys Arg Asn Ser  Asp Lys Leu
    1145                1150                1155

Ile Ala  Arg Lys Lys Asp Trp  Asp Pro Lys Lys Tyr  Gly Gly Phe
    1160                1165                1170

Asp Ser  Pro Thr Val Ala Tyr  Ser Val Leu Val Val  Ala Lys Val
    1175                1180                1185

Glu Lys  Gly Lys Ser Lys Lys  Leu Lys Ser Val Lys  Glu Leu Leu
    1190                1195                1200

Gly Ile  Thr Ile Met Glu Arg  Ser Ser Phe Glu Lys  Asn Pro Ile
    1205                1210                1215

Asp Phe  Leu Glu Ala Lys Gly  Tyr Lys Glu Val Lys  Lys Asp Leu
    1220                1225                1230

Ile Ile  Lys Leu Pro Lys Tyr  Ser Leu Phe Glu Leu  Glu Asn Gly
    1235                1240                1245

Arg Lys  Arg Met Leu Ala Ser  Ala Gly Glu Leu Gln  Lys Gly Asn
    1250                1255                1260

Glu Leu  Ala Leu Pro Ser Lys  Tyr Val Asn Phe Leu  Tyr Leu Ala
    1265                1270                1275

Ser His  Tyr Glu Lys Leu Lys  Gly Ser Pro Glu Asp  Asn Glu Gln
    1280                1285                1290

Lys Gln  Leu Phe Val Glu Gln  His Lys His Tyr Leu  Asp Glu Ile
    1295                1300                1305

Ile Glu  Gln Ile Ser Glu Phe  Ser Lys Arg Val Ile  Leu Ala Asp
    1310                1315                1320

Ala Asn  Leu Asp Lys Val Leu  Ser Ala Tyr Asn Lys  His Arg Asp
    1325                1330                1335

Lys Pro  Ile Arg Glu Gln Ala  Glu Asn Ile Ile His  Leu Phe Thr
    1340                1345                1350

Leu Thr  Asn Leu Gly Ala Pro  Ala Ala Phe Lys Tyr  Phe Asp Thr
    1355                1360                1365

Thr Ile  Asp Arg Lys Arg Tyr  Thr Ser Thr Lys Glu  Val Leu Asp
    1370                1375                1380

Ala Thr  Leu Ile His Gln Ser  Ile Thr Gly Leu Tyr  Glu Thr Arg
    1385                1390                1395

Ile Asp  Leu Ser Gln Leu Gly  Gly Asp Lys Arg Pro  Ala Ala Thr
    1400                1405                1410

Lys Lys  Ala Gly Gln Ala Lys  Lys Lys Lys
    1415                1420
```

<210> SEQ ID NO 51
<211> LENGTH: 2012
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 51

```
gaatgctgcc ctcagacccg cttcctccct gtccttgtct gtccaaggag aatgaggtct     60

cactggtgga tttcggacta ccctgaggag ctggcacctg agggacaagg ccccccacct    120

gcccagctcc agcctctgat gaggggtggg agagagctac atgaggttgc taagaaagcc    180
```

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tcccctgaag gagaccacac agtgtgtgag gttggagtct ctagcagcgg gttctgtgcc 240

cccagggata gtctggctgt ccaggcactg ctcttgatat aaacaccacc tcctagttat 300

gaaaccatgc ccattctgcc tctctgtatg gaaaagagca tggggctggc ccgtggggtg 360

gtgtccactt taggccctgt gggagatcat gggaacccac gcagtgggtc ataggctctc 420

tcatttacta ctcacatcca ctctgtgaag aagcgattat gatctctcct ctagaaactc 480

gtagagtccc atgtctgccg gcttccagag cctgcactcc tccaccttgg cttggctttg 540

ctggggctag aggagctagg atgcacagca gctctgtgac cctttgtttg agaggaacag 600

gaaaaccacc cttctctctg gcccactgtg tcctcttcct gccctgccat ccccttctgt 660

gaatgttaga cccatgggag cagctggtca gaggggaccc cggcctgggg cccctaaccc 720

tatgtagcct cagtcttccc atcaggctct cagctcagcc tgagtgttga ggccccagtg 780

gctgctctgg gggcctcctg agtttctcat ctgtgcccct ccctccctgg cccaggtgaa 840

ggtgtggttc cagaaccgga ggacaaagta caaacggcag aagctggagg aggaagggcc 900

tgagtccgag cagaagaaga agggctccca tcacatcaac cggtggcgca ttgccacgaa 960

gcaggccaat ggggaggaca tcgatgtcac ctccaatgac aagcttgcta gcggtgggca 1020

accacaaacc cacgagggca gagtgctgct tgctgctggc caggcccctg cgtgggccca 1080

agctggactc tggccactcc ctggccaggc tttggggagg cctggagtca tggccccaca 1140

gggcttgaag cccggggccg ccattgacag agggacaagc aatgggctgg ctgaggcctg 1200

ggaccacttg gccttctcct cggagagcct gcctgcctgg gcgggcccgc ccgccaccgc 1260

agcctcccag ctgctctccg tgtctccaat ctccctttg ttttgatgca tttctgtttt 1320

aatttatttt ccaggcacca ctgtagttta gtgatcccca gtgtcccct tccctatggg 1380

aataataaaa gtctctctct taatgacacg ggcatccagc tccagcccca gagcctgggg 1440

tggtagattc cggctctgag ggccagtggg ggctggtaga gcaaacgcgt tcagggcctg 1500

ggagcctggg gtggggtact ggtggagggg gtcaagggta attcattaac tcctctcttt 1560

tgttgggga ccctggtctc tacctccagc tccacagcag gagaaacagg ctagacatag 1620

ggaagggcca tcctgtatct tgagggagga caggcccagg tctttcttaa cgtattgaga 1680

ggtgggaatc aggcccaggt agttcaatgg gagagggaga gtgcttccct ctgcctagag 1740

actctggtgg cttctccagt tgaggagaaa ccagaggaaa ggggaggatt ggggtctggg 1800

ggagggaaca ccattcacaa aggctgacgg ttccagtccg aagtcgtggg cccaccagga 1860

tgctcacctg tccttggaga accgctgggc aggttgagac tgcagagaca gggcttaagg 1920

ctgagcctgc aaccagtccc cagtgactca gggcctcctc agcccaagaa agagcaacgt 1980

gccagggccc gctgagctct tgtgttcacc tg 2012

<210> SEQ ID NO 52
<211> LENGTH: 1153
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polypeptide

<400> SEQUENCE: 52

Met Lys Arg Pro Ala Ala Thr Lys Lys Ala Gly Gln Ala Lys Lys Lys
1 5 10 15

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```
Lys Ser Asp Leu Val Leu Gly Leu Asp Ile Gly Ile Gly Ser Val Gly
            20                  25                  30

Val Gly Ile Leu Asn Lys Val Thr Gly Glu Ile Ile His Lys Asn Ser
        35                  40                  45

Arg Ile Phe Pro Ala Ala Gln Ala Glu Asn Asn Leu Val Arg Arg Thr
    50                  55                  60

Asn Arg Gln Gly Arg Arg Leu Ala Arg Arg Lys Lys His Arg Arg Val
65                  70                  75                  80

Arg Leu Asn Arg Leu Phe Glu Glu Ser Gly Leu Ile Thr Asp Phe Thr
                85                  90                  95

Lys Ile Ser Ile Asn Leu Asn Pro Tyr Gln Leu Arg Val Lys Gly Leu
            100                 105                 110

Thr Asp Glu Leu Ser Asn Glu Glu Leu Phe Ile Ala Leu Lys Asn Met
        115                 120                 125

Val Lys His Arg Gly Ile Ser Tyr Leu Asp Asp Ala Ser Asp Asp Gly
    130                 135                 140

Asn Ser Ser Val Gly Asp Tyr Ala Gln Ile Val Lys Glu Asn Ser Lys
145                 150                 155                 160

Gln Leu Glu Thr Lys Thr Pro Gly Gln Ile Gln Leu Glu Arg Tyr Gln
                165                 170                 175

Thr Tyr Gly Gln Leu Arg Gly Asp Phe Thr Val Glu Lys Asp Gly Lys
            180                 185                 190

Lys His Arg Leu Ile Asn Val Phe Pro Thr Ser Ala Tyr Arg Ser Glu
        195                 200                 205

Ala Leu Arg Ile Leu Gln Thr Gln Gln Glu Phe Asn Pro Gln Ile Thr
    210                 215                 220

Asp Glu Phe Ile Asn Arg Tyr Leu Glu Ile Leu Thr Gly Lys Arg Lys
225                 230                 235                 240

Tyr Tyr His Gly Pro Gly Asn Glu Lys Ser Arg Thr Asp Tyr Gly Arg
                245                 250                 255

Tyr Arg Thr Ser Gly Glu Thr Leu Asp Asn Ile Phe Gly Ile Leu Ile
            260                 265                 270

Gly Lys Cys Thr Phe Tyr Pro Asp Glu Phe Arg Ala Ala Lys Ala Ser
        275                 280                 285

Tyr Thr Ala Gln Glu Phe Asn Leu Leu Asn Asp Leu Asn Asn Leu Thr
    290                 295                 300

Val Pro Thr Glu Thr Lys Lys Leu Ser Lys Glu Gln Lys Asn Gln Ile
305                 310                 315                 320

Ile Asn Tyr Val Lys Asn Glu Lys Ala Met Gly Pro Ala Lys Leu Phe
                325                 330                 335

Lys Tyr Ile Ala Lys Leu Leu Ser Cys Asp Val Ala Asp Ile Lys Gly
            340                 345                 350

Tyr Arg Ile Asp Lys Ser Gly Lys Ala Glu Ile His Thr Phe Glu Ala
        355                 360                 365

Tyr Arg Lys Met Lys Thr Leu Glu Thr Leu Asp Ile Glu Gln Met Asp
    370                 375                 380

Arg Glu Thr Leu Asp Lys Leu Ala Tyr Val Leu Thr Leu Asn Thr Glu
385                 390                 395                 400

Arg Glu Gly Ile Gln Glu Ala Leu Glu His Glu Phe Ala Asp Gly Ser
                405                 410                 415

Phe Ser Gln Lys Gln Val Asp Glu Leu Val Gln Phe Arg Lys Ala Asn
            420                 425                 430
```

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```
Ser Ser Ile Phe Gly Lys Gly Trp His Asn Phe Ser Val Lys Leu Met
        435                 440                 445

Met Glu Leu Ile Pro Glu Leu Tyr Glu Thr Ser Glu Glu Gln Met Thr
    450                 455                 460

Ile Leu Thr Arg Leu Gly Lys Gln Lys Thr Thr Ser Ser Ser Asn Lys
465                 470                 475                 480

Thr Lys Tyr Ile Asp Glu Lys Leu Leu Thr Glu Glu Ile Tyr Asn Pro
                485                 490                 495

Val Val Ala Lys Ser Val Arg Gln Ala Ile Lys Ile Val Asn Ala Ala
            500                 505                 510

Ile Lys Glu Tyr Gly Asp Phe Asp Asn Ile Val Ile Glu Met Ala Arg
        515                 520                 525

Glu Thr Asn Glu Asp Asp Glu Lys Lys Ala Ile Gln Lys Ile Gln Lys
    530                 535                 540

Ala Asn Lys Asp Glu Lys Asp Ala Ala Met Leu Lys Ala Ala Asn Gln
545                 550                 555                 560

Tyr Asn Gly Lys Ala Glu Leu Pro His Ser Val Phe His Gly His Lys
                565                 570                 575

Gln Leu Ala Thr Lys Ile Arg Leu Trp His Gln Gln Gly Glu Arg Cys
            580                 585                 590

Leu Tyr Thr Gly Lys Thr Ile Ser Ile His Asp Leu Ile Asn Asn Ser
        595                 600                 605

Asn Gln Phe Glu Val Asp His Ile Leu Pro Leu Ser Ile Thr Phe Asp
    610                 615                 620

Asp Ser Leu Ala Asn Lys Val Leu Val Tyr Ala Thr Ala Asn Gln Glu
625                 630                 635                 640

Lys Gly Gln Arg Thr Pro Tyr Gln Ala Leu Asp Ser Met Asp Asp Ala
                645                 650                 655

Trp Ser Phe Arg Glu Leu Lys Ala Phe Val Arg Glu Ser Lys Thr Leu
            660                 665                 670

Ser Asn Lys Lys Lys Glu Tyr Leu Leu Thr Glu Glu Asp Ile Ser Lys
        675                 680                 685

Phe Asp Val Arg Lys Lys Phe Ile Glu Arg Asn Leu Val Asp Thr Arg
    690                 695                 700

Tyr Ala Ser Arg Val Val Leu Asn Ala Leu Gln Glu His Phe Arg Ala
705                 710                 715                 720

His Lys Ile Asp Thr Lys Val Ser Val Val Arg Gly Gln Phe Thr Ser
                725                 730                 735

Gln Leu Arg Arg His Trp Gly Ile Glu Lys Thr Arg Asp Thr Tyr His
            740                 745                 750

His His Ala Val Asp Ala Leu Ile Ile Ala Ala Ser Ser Gln Leu Asn
        755                 760                 765

Leu Trp Lys Lys Gln Lys Asn Thr Leu Val Ser Tyr Ser Glu Asp Gln
    770                 775                 780

Leu Leu Asp Ile Glu Thr Gly Glu Leu Ile Ser Asp Asp Glu Tyr Lys
785                 790                 795                 800

Glu Ser Val Phe Lys Ala Pro Tyr Gln His Phe Val Asp Thr Leu Lys
                805                 810                 815

Ser Lys Glu Phe Glu Asp Ser Ile Leu Phe Ser Tyr Gln Val Asp Ser
            820                 825                 830

Lys Phe Asn Arg Lys Ile Ser Asp Ala Thr Ile Tyr Ala Thr Arg Gln
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```
        835                 840                 845

Ala Lys Val Gly Lys Asp Lys Ala Asp Glu Thr Tyr Val Leu Gly Lys
    850                 855                 860

Ile Lys Asp Ile Tyr Thr Gln Asp Gly Tyr Asp Ala Phe Met Lys Ile
865                 870                 875                 880

Tyr Lys Lys Asp Lys Ser Lys Phe Leu Met Tyr Arg His Asp Pro Gln
                885                 890                 895

Thr Phe Glu Lys Val Ile Glu Pro Ile Leu Glu Asn Tyr Pro Asn Lys
            900                 905                 910

Gln Ile Asn Glu Lys Gly Lys Glu Val Pro Cys Asn Pro Phe Leu Lys
        915                 920                 925

Tyr Lys Glu Glu His Gly Tyr Ile Arg Lys Tyr Ser Lys Lys Gly Asn
    930                 935                 940

Gly Pro Glu Ile Lys Ser Leu Lys Tyr Tyr Asp Ser Lys Leu Gly Asn
945                 950                 955                 960

His Ile Asp Ile Thr Pro Lys Asp Ser Asn Asn Lys Val Val Leu Gln
                965                 970                 975

Ser Val Ser Pro Trp Arg Ala Asp Val Tyr Phe Asn Lys Thr Thr Gly
            980                 985                 990

Lys Tyr Glu Ile Leu Gly Leu Lys  Tyr Ala Asp Leu Gln  Phe Glu Lys
        995                 1000                1005

Gly Thr  Gly Thr Tyr Lys Ile  Ser Gln Glu Lys Tyr  Asn Asp Ile
    1010                1015                1020

Lys Lys  Lys Glu Gly Val Asp  Ser Asp Ser Glu Phe  Lys Phe Thr
    1025                1030                1035

Leu Tyr  Lys Asn Asp Leu Leu  Leu Val Lys Asp Thr  Glu Thr Lys
    1040                1045                1050

Glu Gln  Gln Leu Phe Arg Phe  Leu Ser Arg Thr Met  Pro Lys Gln
    1055                1060                1065

Lys His  Tyr Val Glu Leu Lys  Pro Tyr Asp Lys Gln  Lys Phe Glu
    1070                1075                1080

Gly Gly  Glu Ala Leu Ile Lys  Val Leu Gly Asn Val  Ala Asn Ser
    1085                1090                1095

Gly Gln  Cys Lys Lys Gly Leu  Gly Lys Ser Asn Ile  Ser Ile Tyr
    1100                1105                1110

Lys Val  Arg Thr Asp Val Leu  Gly Asn Gln His Ile  Ile Lys Asn
    1115                1120                1125

Glu Gly  Asp Lys Pro Lys Leu  Asp Phe Lys Arg Pro  Ala Ala Thr
    1130                1135                1140

Lys Lys  Ala Gly Gln Ala Lys  Lys Lys Lys
    1145                1150


<210> SEQ ID NO 53
<211> LENGTH: 340
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polynucleotide

<400> SEQUENCE: 53

gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc tgttagagag     60

ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga    120
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aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccg ttacttaaat cttgcagaag ctacaaagat aaggcttcat gccgaaatca 300

acaccctgtc attttatggc agggtgtttt cgttatttaa 340

<210> SEQ ID NO 54
<211> LENGTH: 360
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (288)..(317)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 54

gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc tgttagagag 60

ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga 120

aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccg ggttttagag ctatgctgtt ttgaatggtc ccaaaacnnn nnnnnnnnnn 300

nnnnnnnnnn nnnnnnngtt ttagagctat gctgttttga atggtcccaa aacttttttt 360

<210> SEQ ID NO 55
<211> LENGTH: 318
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (250)..(269)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 55

gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc tgttagagag 60

ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga 120

aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccn nnnnnnnnnn nnnnnnnnng ttttagagct agaaatagca agttaaaata 300

aggctagtcc gttttttt 318

<210> SEQ ID NO 56
<211> LENGTH: 325
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (250)..(269)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 56

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gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc tgttagagag 60

ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga 120

aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccn nnnnnnnnnn nnnnnnnnng ttttagagct agaaatagca agttaaaata 300

aggctagtcc gttatcattt ttttt 325

<210> SEQ ID NO 57
<211> LENGTH: 337
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (250)..(269)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 57

gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc tgttagagag 60

ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga 120

aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccn nnnnnnnnnn nnnnnnnnng ttttagagct agaaatagca agttaaaata 300

aggctagtcc gttatcaact tgaaaaagtg ttttttt 337

<210> SEQ ID NO 58
<211> LENGTH: 352
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (250)..(269)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 58

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ataattggaa ttaatttgac tgtaaacaca aagatattag tacaaaatac gtgacgtaga 120

aagtaataat ttcttgggta gtttgcagtt ttaaaattat gttttaaaat ggactatcat 180

atgcttaccg taacttgaaa gtatttcgat ttcttggctt tatatatctt gtggaaagga 240

cgaaacaccn nnnnnnnnnn nnnnnnnnng ttttagagct agaaatagca agttaaaata 300

aggctagtcc gttatcaact tgaaaaagtg gcaccgagtc ggtgcttttt tt 352

<210> SEQ ID NO 59
<211> LENGTH: 5101
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 59

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cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc cccgcccatt 60

gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca 120

atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc 180

aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta 240

catgaccttа tgggactttc ctacttggca gtacatctac gtattagtca tcgctattac 300

catggtcgag gtgagcccca cgttctgctt cactctcccc atctcccccc cctccccacc 360

cccaattttg tatttattta ttttttaatt attttgtgca gcgatggggg cgggggggg 420

ggggggcgc gcgccaggcg gggcggggcg gggcgagggg cggggcgggg cgaggcggag 480

aggtgcggcg gcagccaatc agagcggcgc gctccgaaag tttcctttta tggcgaggcg 540

gcggcggcgg cggccctata aaaagcgaag cgcgcggcgg gcgggagtcg ctgcgacgct 600

gccttcgccc cgtgccccgc tccgccgccg cctcgcgccg cccgccccgg ctctgactga 660

ccgcgttact cccacaggtg agcgggcggg acggcccttc tcctccgggc tgtaattagc 720

tgagcaagag gtaagggttt aagggatggt tggttggtgg ggtattaatg tttaattacc 780

tggagcacct gcctgaaatc actttttttc aggttggacc ggtgccacca tggactataa 840

ggaccacgac ggagactaca aggatcatga tattgattac aaagacgatg acgataagat 900

ggccccaaag aagaagcgga aggtcggtat ccacggagtc ccagcagccg acaagaagta 960

cagcatcggc ctggacatcg gcaccaactc tgtgggctgg gccgtgatca ccgacgagta 1020

caaggtgccc agcaagaaat tcaaggtgct gggcaacacc gaccggcaca gcatcaagaa 1080

gaacctgatc ggagccctgc tgttcgacag cggcgaaaca gccgaggcca cccggctgaa 1140

gagaaccgcc agaagaagat acaccagacg gaagaaccgg atctgctatc tgcaagagat 1200

cttcagcaac gagatggcca aggtggacga cagcttcttc cacagactgg aagagtcctt 1260

cctggtggaa gaggataaga agcacgagcg gcaccccatc ttcggcaaca tcgtggacga 1320

ggtggcctac cacgagaagt accccaccat ctaccacctg agaaagaaac tggtggacag 1380

caccgacaag gccgacctgc ggctgatcta tctggccctg gcccacatga tcaagttccg 1440

gggccacttc ctgatcgagg gcgacctgaa ccccgacaac agcgacgtgg acaagctgtt 1500

catccagctg gtgcagacct acaaccagct gttcgaggaa aaccccatca acgccagcgg 1560

cgtggacgcc aaggccatcc tgtctgccag actgagcaag agcagacggc tggaaaatct 1620

gatcgcccag ctgcccggcg agaagaagaa tggcctgttc ggcaacctga ttgccctgag 1680

cctgggcctg accccaact tcaagagcaa cttcgacctg gccgaggatg ccaaactgca 1740

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gtacgccgac ctgtttctgg ccgccaagaa cctgtccgac gccatcctgc tgagcgacat 1860

cctgagagtg aacaccgaga tcaccaaggc ccccctgagc gcctctatga tcaagagata 1920

cgacgagcac caccaggacc tgaccctgct gaaagctctc gtgcggcagc agctgcctga 1980

gaagtacaaa gagattttct tcgaccagag caagaacggc tacgccggct acattgacgg 2040

cggagccagc caggaagagt tctacaagtt catcaagccc atcctggaaa agatggacgg 2100

caccgaggaa ctgctcgtga agctgaacag agaggacctg ctgcggaagc agcggacctt 2160

cgacaacggc agcatccccc accagatcca cctgggagag ctgcacgcca ttctgcggcg 2220

gcaggaagat ttttacccat tcctgaagga caaccgggaa aagatcgaga agatcctgac 2280

cttccgcatc ccctactacg tgggccctct ggccagggga aacagcagat tcgcctggat 2340

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gaccagaaag agcgaggaaa ccatcacccc ctggaacttc gaggaagtgg tggacaaggg 2400

cgcttccgcc cagagcttca tcgagcggat gaccaacttc gataagaacc tgcccaacga 2460

gaaggtgctg cccaagcaca gcctgctgta cgagtacttc accgtgtata acgagctgac 2520

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agaggactac ttcaagaaaa tcgagtgctt cgactccgtg gaaatctccg gcgtggaaga 2700

tcggttcaac gcctccctgg gcacatacca cgatctgctg aaaattatca aggacaagga 2760

cttcctggac aatgaggaaa acgaggacat tctggaagat atcgtgctga ccctgacact 2820

gtttgaggac agagagatga tcgaggaacg gctgaaaacc tatgcccacc tgttcgacga 2880

caaagtgatg aagcagctga agcggcggag atacaccggc tggggcaggc tgagccggaa 2940

gctgatcaac ggcatccggg acaagcagtc cggcaagaca atcctggatt tcctgaagtc 3000

cgacggcttc gccaacagaa acttcatgca gctgatccac gacgacagcc tgacctttaa 3060

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cgagctcgtg aaagtgatgg gccggcacaa gcccgagaac atcgtgatcg aaatggccag 3240

agagaaccag accacccaga agggacagaa gaacagccgc gagagaatga agcggatcga 3300

agagggcatc aaagagctgg gcagccagat cctgaaagaa caccccgtgg aaaacaccca 3360

gctgcagaac gagaagctgt acctgtacta cctgcagaat gggcgggata tgtacgtgga 3420

ccaggaactg gacatcaacc ggctgtccga ctacgatgtg gaccatatcg tgcctcagag 3480

ctttctgaag gacgactcca tcgacaacaa ggtgctgacc agaagcgaca agaaccgggg 3540

caagagcgac aacgtgccct ccgaagaggt cgtgaagaag atgaagaact actggcggca 3600

gctgctgaac gccaagctga ttacccagag aaagttcgac aatctgacca aggccgagag 3660

aggcggcctg agcgaactgg ataaggccgg cttcatcaag agacagctgg tggaaacccg 3720

gcagatcaca aagcacgtgg cacagatcct ggactcccgg atgaacacta agtacgacga 3780

gaatgacaag ctgatccggg aagtgaaagt gatcaccctg aagtccaagc tggtgtccga 3840

tttccggaag gatttccagt tttacaaagt gcgcgagatc aacaactacc accacgccca 3900

cgacgcctac ctgaacgccg tcgtgggaac cgccctgatc aaaaagtacc ctaagctgga 3960

aagcgagttc gtgtacggcg actacaaggt gtacgacgtg cggaagatga tcgccaagag 4020

cgagcaggaa atcggcaagg ctaccgccaa gtacttcttc tacagcaaca tcatgaactt 4080

tttcaagacc gagattaccc tggccaacgg cgagatccgg aagcggcctc tgatcgagac 4140

aaacggcgaa accggggaga tcgtgtggga taagggccgg gattttgcca ccgtgcggaa 4200

agtgctgagc atgccccaag tgaatatcgt gaaaaagacc gaggtgcaga caggcggctt 4260

cagcaaagag tctatcctgc ccaagaggaa cagcgataag ctgatcgcca gaaagaagga 4320

ctgggaccct aagaagtacg gcggcttcga cagccccacc gtggcctatt ctgtgctggt 4380

ggtggccaaa gtggaaaagg gcaagtccaa gaaactgaag agtgtgaaag agctgctggg 4440

gatcaccatc atggaaagaa gcagcttcga gaagaatccc atcgactttc tggaagccaa 4500

gggctacaaa gaagtgaaaa aggacctgat catcaagctg cctaagtact ccctgttcga 4560

gctggaaaac ggccggaaga gaatgctggc ctctgccggc gaactgcaga agggaaacga 4620

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actggccctg ccctccaaat atgtgaactt cctgtacctg gccagccact atgagaagct 4680

gaagggctcc cccgaggata atgagcagaa acagctgttt gtggaacagc acaagcacta 4740

cctggacgag atcatcgagc agatcagcga gttctccaag agagtgatcc tggccgacgc 4800

taatctggac aaagtgctgt ccgcctacaa caagcaccgg gataagccca tcagagagca 4860

ggccgagaat atcatccacc tgtttaccct gaccaatctg ggagcccctg ccgccttcaa 4920

gtactttgac accaccatcg accggaagag gtacaccagc accaaagagg tgctggacgc 4980

caccctgatc caccagagca tcaccggcct gtacgagaca cggatcgacc tgtctcagct 5040

gggaggcgac tttctttttc ttagcttgac cagctttctt agtagcagca ggacgcttta 5100

a 5101

<210> SEQ ID NO 60
<211> LENGTH: 137
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 60

nnnnnnnnnn nnnnnnnnnn gttattgtac tctcaagatt tagaaataaa tcttgcagaa 60

gctacaaaga taaggcttca tgccgaaatc aacaccctgt cattttatgg cagggtgttt 120

tcgttattta atttttt 137

<210> SEQ ID NO 61
<211> LENGTH: 123
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 61

nnnnnnnnnn nnnnnnnnnn gttattgtac tctcagaaat gcagaagcta caaagataag 60

gcttcatgcc gaaatcaaca ccctgtcatt ttatggcagg gtgttttcgt tatttaattt 120

ttt 123

<210> SEQ ID NO 62
<211> LENGTH: 110
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 62

nnnnnnnnnn nnnnnnnnnn gttattgtac tctcagaaat gcagaagcta caaagataag 60

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gcttcatgcc gaaatcaaca ccctgtcatt ttatggcagg gtgttttttt 110

<210> SEQ ID NO 63
<211> LENGTH: 137
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 63

nnnnnnnnnn nnnnnnnnnn gttattgtac tctcaagatt tagaaataaa tcttgcagaa 60

gctacaatga taaggcttca tgccgaaatc aacaccctgt cattttatgg cagggtgttt 120

tcgttattta atttttt 137

<210> SEQ ID NO 64
<211> LENGTH: 123
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 64

nnnnnnnnnn nnnnnnnnnn gttattgtac tctcagaaat gcagaagcta caatgataag 60

gcttcatgcc gaaatcaaca ccctgtcatt ttatggcagg gtgttttcgt tatttaattt 120

ttt 123

<210> SEQ ID NO 65
<211> LENGTH: 110
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 65

nnnnnnnnnn nnnnnnnnnn gttattgtac tctcagaaat gcagaagcta caatgataag 60

gcttcatgcc gaaatcaaca ccctgtcatt ttatggcagg gtgttttttt 110

<210> SEQ ID NO 66
<211> LENGTH: 107
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 66

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nnnnnnnnnn nnnnnnnnnn gttttagagc tgtggaaaca cagcgagtta aaataaggct 60

tagtccgtac tcaacttgaa aaggtggcac cgattcggtg ttttttt 107

<210> SEQ ID NO 67
<211> LENGTH: 4263
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 67

atgaaaaggc cggcggccac gaaaaaggcc ggccaggcaa aaaagaaaaa gaccaagccc 60

tacagcatcg gcctggacat cggcaccaat agcgtgggct gggccgtgac caccgacaac 120

tacaaggtgc ccagcaagaa aatgaaggtg ctgggcaaca cctccaagaa gtacatcaag 180

aaaaacctgc tgggcgtgct gctgttcgac agcggcatta cagccgaggg cagacggctg 240

aagagaaccg ccagacggcg gtacacccgg cggagaaaca gaatcctgta tctgcaagag 300

atcttcagca ccgagatggc taccctggac gacgccttct tccagcggct ggacgacagc 360

ttcctggtgc ccgacgacaa gcgggacagc aagtacccca tcttcggcaa cctggtggaa 420

gagaaggcct accacgacga gttccccacc atctaccacc tgagaaagta cctggccgac 480

agcaccaaga aggccgacct gagactggtg tatctggccc tggcccacat gatcaagtac 540

cggggccact tcctgatcga gggcgagttc aacagcaaga acaacgacat ccagaagaac 600

ttccaggact tcctggacac ctacaacgcc atcttcgaga gcgacctgtc cctggaaaac 660

agcaagcagc tggaagagat cgtgaaggac aagatcagca agctggaaaa gaaggaccgc 720

atcctgaagc tgttccccgg cgagaagaac agcggaatct tcagcgagtt tctgaagctg 780

atcgtgggca accaggccga cttcagaaag tgcttcaacc tggacgagaa agccagcctg 840

cacttcagca aagagagcta cgacgaggac ctggaaaccc tgctgggata tatcggcgac 900

gactacagcg acgtgttcct gaaggccaag aagctgtacg acgctatcct gctgagcggc 960

ttcctgaccg tgaccgacaa cgagacagag gccccactga gcagcgccat gattaagcgg 1020

tacaacgagc acaaagagga tctggctctg ctgaaagagt acatccggaa catcagcctg 1080

aaaacctaca atgaggtgtt caaggacgac accaagaacg gctacgccgg ctacatcgac 1140

ggcaagacca accaggaaga tttctatgtg tacctgaaga agctgctggc cgagttcgag 1200

ggggccgact actttctgga aaaaatcgac cgcgaggatt tcctgcggaa gcagcggacc 1260

ttcgacaacg gcagcatccc ctaccagatc catctgcagg aaatgcgggc catcctggac 1320

aagcaggcca agttctaccc attcctggcc aagaacaaag agcggatcga gaagatcctg 1380

accttccgca tcccttacta cgtgggcccc ctggccagag gcaacagcga ttttgcctgg 1440

tccatccgga agcgcaatga gaagatcacc ccctggaact tcgaggacgt gatcgacaaa 1500

gagtccagcg ccgaggcctt catcaaccgg atgaccagct tcgacctgta cctgcccgag 1560

gaaaaggtgc tgcccaagca cagcctgctg tacgagacat tcaatgtgta taacgagctg 1620

accaaagtgc ggtttatcgc cgagtctatg cgggactacc agttcctgga ctccaagcag 1680

aaaaaggaca tcgtgcggct gtacttcaag gacaagcgga aagtgaccga taaggacatc 1740

atcgagtacc tgcacgccat ctacggctac gatggcatcg agctgaaggg catcgagaag 1800

cagttcaact ccagcctgag cacataccac gacctgctga acattatcaa cgacaaagaa 1860

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tttctggacg actccagcaa cgaggccatc atcgaagaga tcatccacac cctgaccatc 1920

tttgaggacc gcgagatgat caagcagcgg ctgagcaagt tcgagaacat cttcgacaag 1980

agcgtgctga aaaagctgag cagacggcac tacaccggct ggggcaagct gagcgccaag 2040

ctgatcaacg gcatccggga cgagaagtcc ggcaacacaa tcctggacta cctgatcgac 2100

gacggcatca gcaaccggaa cttcatgcag ctgatccacg acgacgccct gagcttcaag 2160

aagaagatcc agaaggccca gatcatcggg gacgaggaca agggcaacat caaagaagtc 2220

gtgaagtccc tgcccggcag ccccgccatc aagaagggaa tcctgcagag catcaagatc 2280

gtggacgagc tcgtgaaagt gatgggcggc agaaagcccg agagcatcgt ggtggaaatg 2340

gctagagaga accagtacac caatcagggc aagagcaaca gccagcagag actgaagaga 2400

ctggaaaagt ccctgaaaga gctgggcagc aagattctga aagagaatat ccctgccaag 2460

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aatggcaagg acatgtatac aggcgacgac ctggatatcg accgcctgag caactacgac 2580

atcgaccata ttatccccca ggccttcctg aaagacaaca gcattgacaa caaagtgctg 2640

gtgtcctccg ccagcaaccg cggcaagtcc gatgatgtgc ccagcctgga agtcgtgaaa 2700

aagagaaaga ccttctggta tcagctgctg aaaagcaagc tgattagcca gaggaagttc 2760

gacaacctga ccaaggccga gaggaggcggc ctgagccctg aagataaggc cggcttcatc 2820

cagagacagc tggtggaaac ccggcagatc accaagcacg tggccagact gctggatgag 2880

aagtttaaca acaagaagga cgagaacaac cgggccgtgc ggaccgtgaa gatcatcacc 2940

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cgggtgctga gttatcctca agtgaatgtc gtgaagaagg tggaagaaca gaaccacggc 3360

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agccagaaat ttgtgaaact gctgtaccac gccaagcgga tctccaacac catcaatgag 3840

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ggcagcgagc ggaagggact gtttgagctg acctccagag gctctgccgc cgactttgag 4080

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gccaccctga tccaccagag cgtgaccggc ctgtacgaaa cccggatcga cctggctaag 4200

ctgggcgagg gaaagcgtcc tgctgctact aagaaagctg gtcaagctaa gaaaaagaaa 4260

taa 4263

<210> SEQ ID NO 68
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 68

tcctagcagg atttctgata ttactgtcac gttttagagc tatgctgttt tga 53

<210> SEQ ID NO 69
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 69

gtgacagtaa tatcagaaat cctgctagga gttttgggac cattcaaaac agc 53

<210> SEQ ID NO 70
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 70

gggtttcaag tctttgtagc aagag 25

<210> SEQ ID NO 71
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 71

gccaatgaac gggaaccctt ggtc 24

<210> SEQ ID NO 72
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(4)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 72

nnnngacgag gcaatggctg aaatc 25

<210> SEQ ID NO 73

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<211> LENGTH: 25
<212> TYPE: DNA
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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
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<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(4)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 73

nnnnttattt ggctcatatt tgctg 25

<210> SEQ ID NO 74
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 74

ctttacacca atcgctgcaa cagac 25

<210> SEQ ID NO 75
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 75

caaaatttct agtcttcttt gcctttcccc ataaaaccct cctta 45

<210> SEQ ID NO 76
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 76

agggttttat ggggaaaggc aaagaagact agaaattttg atacc 45

<210> SEQ ID NO 77
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 77

cttacggtgc ataaagtcaa tttcc 25

<210> SEQ ID NO 78
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 78

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tggctcgatt tcagccattg c 21

<210> SEQ ID NO 79
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (33)..(33)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 79

ctttgacgag gcaatggctg aaatcgagcc aanaaagcgc aag 43

<210> SEQ ID NO 80
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (34)..(34)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 80

ctttgacgag gcaatggctg aaatcgagcc aaanaagcgc aag 43

<210> SEQ ID NO 81
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (35)..(35)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 81

ctttgacgag gcaatggctg aaatcgagcc aaaanagcgc aag 43

<210> SEQ ID NO 82
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
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<222> LOCATION: (36)..(36)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 82

ctttgacgag gcaatggctg aaatcgagcc aaaaangcgc aag 43

<210> SEQ ID NO 83
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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
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<222> LOCATION: (37)..(37)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 83

ctttgacgag gcaatggctg aaatcgagcc aaaaaancgc aag 43

<210> SEQ ID NO 84
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (38)..(38)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 84

ctttgacgag gcaatggctg aaatcgagcc aaaaaagngc aag 43

<210> SEQ ID NO 85
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (39)..(39)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 85

ctttgacgag gcaatggctg aaatcgagcc aaaaaagcnc aagaag 46

<210> SEQ ID NO 86
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (40)..(40)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 86

ctttgacgag gcaatggctg aaatcgagcc aaaaaagcgn aagaag 46

<210> SEQ ID NO 87
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (41)..(41)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 87

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ctttgacgag gcaatggctg aaatcgagcc aaaaaagcgc nagaag 46

<210> SEQ ID NO 88
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 88

gcgctttttt ggctcgattt cag 23

<210> SEQ ID NO 89
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
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<222> LOCATION: (31)..(31)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 89

caatggctga aatcgagcca aaaaagcgca ngaagaaatc 40

<210> SEQ ID NO 90
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (32)..(32)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 90

caatggctga aatcgagcca aaaaagcgca anaagaaatc 40

<210> SEQ ID NO 91
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (33)..(33)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 91

caatggctga aatcgagcca aaaaagcgca agnagaaatc 40

<210> SEQ ID NO 92
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
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<222> LOCATION: (34)..(34)

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<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 92

caatggctga aatcgagcca aaaaagcgca agangaaatc 40

<210> SEQ ID NO 93
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (35)..(35)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 93

caatggctga aatcgagcca aaaaagcgca agaanaaatc 40

<210> SEQ ID NO 94
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (36)..(36)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 94

caatggctga aatcgagcca aaaaagcgca agaagnaatc aacc 44

<210> SEQ ID NO 95
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (37)..(37)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 95

caatggctga aatcgagcca aaaaagcgca agaaganatc aacc 44

<210> SEQ ID NO 96
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (38)..(38)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 96

caatggctga aatcgagcca aaaaagcgca agaagaantc aacc 44

<210> SEQ ID NO 97
<211> LENGTH: 44

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (39)..(39)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 97

caatggctga aatcgagcca aaaaagcgca agaagaaanc aacc 44

<210> SEQ ID NO 98
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (40)..(40)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 98

caatggctga aatcgagcca aaaaagcgca agaagaaatn aaccagc 47

<210> SEQ ID NO 99
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (41)..(41)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 99

caatggctga aatcgagcca aaaaagcgca agaagaaatc naccagc 47

<210> SEQ ID NO 100
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 100

gatcctccat ccgtacaacc cacaaccctg g 31

<210> SEQ ID NO 101
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 101

aattccaggg ttgtgggttg tacggatgga g 31

<210> SEQ ID NO 102
<211> LENGTH: 34

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 102

catggatcct atttcttaat aactaaaaat atgg 34

<210> SEQ ID NO 103
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 103

catgaattca actcaacaag tctcagtgtg ctg 33

<210> SEQ ID NO 104
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 104

aaacattttt tctccattta ggaaaaagga tgctg 35

<210> SEQ ID NO 105
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 105

aaaacagcat ccttttcct aaatggagaa aaaat 35

<210> SEQ ID NO 106
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 106

aaaccttaaa tcagtcacaa atagcagcaa aattg 35

<210> SEQ ID NO 107
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 107

aaaacaattt tgctgctatt tgtgactgat ttaag 35

<210> SEQ ID NO 108

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<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 108

aaacttttca tcatacgacc aatctgcttt atttg 35

<210> SEQ ID NO 109
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 109

aaaacaaata aagcagattg gtcgtatgat gaaaa 35

<210> SEQ ID NO 110
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

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aaactcgtcc agaagttatc gtaaaagaaa tcgag 35

<210> SEQ ID NO 111
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 111

aaaactcgat ttcttttacg ataacttctg gacga 35

<210> SEQ ID NO 112
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 112

aaacaatctc tccaaggttt ccttaaaaat ctctg 35

<210> SEQ ID NO 113
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 113

aaaacagaga tttttaagga aaccttggag agatt 35

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<210> SEQ ID NO 114
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 114

aaacgccatc gtcaggaaga agctatgctt gagtg 35

<210> SEQ ID NO 115
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 115

aaaacactca agcatagctt cttcctgacg atggc 35

<210> SEQ ID NO 116
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 116

aaacatctct atacttattg aaatttcttt gtatg 35

<210> SEQ ID NO 117
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 117

aaaacataca aagaaatttc aataagtata gagat 35

<210> SEQ ID NO 118
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 118

aaactagctg tgatagtccg caaaaccagc cttcg 35

<210> SEQ ID NO 119
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 119

aaaacgaagg ctggttttgc ggactatcac agcta 35

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<210> SEQ ID NO 120
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 120

aaacatcgga aggtcgagca agtaattatc ttttg 35

<210> SEQ ID NO 121
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 121

aaaacaaaag ataattactt gctcgacctt ccgat 35

<210> SEQ ID NO 122
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 122

aaacaagatg gtatcgcaaa gtaagtgaca ataag 35

<210> SEQ ID NO 123
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 123

aaaacttatt gtcacttact ttgcgatacc atctt 35

<210> SEQ ID NO 124
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 124

gagacctttg agcttccgag actggtctca gttttgggac cattcaaaac ag 52

<210> SEQ ID NO 125
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 125

tgagaccagt ctcggaagct caaaggtctc gttttagagc tatgctgttt tg 52

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<210> SEQ ID NO 126
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 126

aaactacttt acgcagcgcg gagttcggtt ttttg 35

<210> SEQ ID NO 127
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 127

aaaacaaaaa accgaactcc gcgctgcgta aagta 35

<210> SEQ ID NO 128
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 128

atgccggtac tgccgggcct cttgcgggat tacgaaatca tcctg 45

<210> SEQ ID NO 129
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 129

gtgactggcg atgctgtcgg aatggacgat cacactactc ttctt 45

<210> SEQ ID NO 130
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 130

ttaagaaata atcttcatct aaaatatact tcagtcacct cctagctgac 50

<210> SEQ ID NO 131
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 131

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attgatttga gtcagctagg aggtgactga agtatatttt agatgaag 48

<210> SEQ ID NO 132
<211> LENGTH: 85
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 132

gagacctttg agcttccgag actggtctca gttttgggac cattcaaaac agcatagctc 60

taaaacctcg tagactattt ttgtc 85

<210> SEQ ID NO 133
<211> LENGTH: 84
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 133

gagaccagtc tcggaagctc aaaggtctcg ttttagagct atgctgtttt gaatggtccc 60

aaaacttcag cacactgaga cttg 84

<210> SEQ ID NO 134
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 134

agtcatccca gcaacaaatg g 21

<210> SEQ ID NO 135
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 135

cgtggtaaat cggataacgt tccaagtgaa g 31

<210> SEQ ID NO 136
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 136

tgctcttctt cacaaacaag gg 22

<210> SEQ ID NO 137
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 137

aagccaaagt ttggcaccac c 21

<210> SEQ ID NO 138
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 138

gtagcttatt cagtcctagt gg 22

<210> SEQ ID NO 139
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 139

cgtttgttga actaatgggt gcaaattacg aatcttctcc tgacg 45

<210> SEQ ID NO 140
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 140

cgtcaggaga agattcgtaa tttgcaccca ttagttcaac aaacg 45

<210> SEQ ID NO 141
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 141

gatattatgg agcctatttt tgtgggtttt taggcataaa actatatg 48

<210> SEQ ID NO 142
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 142

catatagttt tatgcctaaa aacccacaaa aataggctcc ataatatc 48

<210> SEQ ID NO 143
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 143

attatttctt aataactaaa aatatgg 27

<210> SEQ ID NO 144
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 144

cgtgtacaat tgctagcgta cggc 24

<210> SEQ ID NO 145
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 145

gcaccggtga tcactagtcc tagg 24

<210> SEQ ID NO 146
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 146

cctaggacta gtgatcaccg gtgcaaatat gagccaaata aatatat 47

<210> SEQ ID NO 147
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 147

gccgtacgct agcaattgta cacgtttgtt gaactaatgg gtgc 44

<210> SEQ ID NO 148
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 148

ttcaaatttt cccatttgat tctcc 25

<210> SEQ ID NO 149
<211> LENGTH: 47
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 149

ccatattttt agttattaag aaataatacc agccatcagt cacctcc 47

<210> SEQ ID NO 150
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 150

agacgattca atagacaata agg 23

<210> SEQ ID NO 151
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 151

gttttgggac cattcaaaac agcatagctc taaaacctcg tagac 45

<210> SEQ ID NO 152
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 152

gctatgctgt tttgaatggt cccaaaacca ttattttaac acacgaggtg 50

<210> SEQ ID NO 153
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 153

gctatgctgt tttgaatggt cccaaaacgc acccattagt tcaacaaacg 50

<210> SEQ ID NO 154
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 154

aattcttttc ttcatcatcg gtc 23

<210> SEQ ID NO 155
<211> LENGTH: 24

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 155

aagaaagaat gaagattgtt catg 24

<210> SEQ ID NO 156
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 156

ggtactaatc aaaatagtga ggagg 25

<210> SEQ ID NO 157
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 157

gtttttcaaa atctgcggtt gcg 23

<210> SEQ ID NO 158
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 158

aaaaattgaa aaaatggtgg aaacac 26

<210> SEQ ID NO 159
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 159

atttcgtaaa cggtatcggt ttcttttaaa gttttgggac cattcaaaac agc 53

<210> SEQ ID NO 160
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 160

tttaaaagaa accgataccg tttacgaaat gttttagagc tatgctgttt tga 53

<210> SEQ ID NO 161

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<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 161

aaacggtatc ggtttctttt aaattcaatt gttttgggac cattcaaaac agc 53

<210> SEQ ID NO 162
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 162

aattgaattt aaaagaaacc gataccgttt gttttagagc tatgctgttt tga 53

<210> SEQ ID NO 163
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 163

gttccttaaa ccaaaacggt atcggtttct tttaaattc 39

<210> SEQ ID NO 164
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 164

gaaaccgata ccgttttggt ttaaggaaca ggtaaagggc atttaac 47

<210> SEQ ID NO 165
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 165

cgatttcagc cattgcctcg tc 22

<210> SEQ ID NO 166
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (29)..(33)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 166

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gcctttgacg aggcaatggc tgaaatcgnn nnnaaaaagc gcaagaagaa atcaac 56

<210> SEQ ID NO 167
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 167

tccgtacaac ccacaaccct gctagtgagc gttttgggac cattcaaaac agc 53

<210> SEQ ID NO 168
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 168

gctcactagc agggttgtgg gttgtacgga gttttagagc tatgctgttt tga 53

<210> SEQ ID NO 169
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 169

ttgttgccac tcttccttct ttc 23

<210> SEQ ID NO 170
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 170

cagggttgtg ggttgttgcg atggagttaa ctcccatctc c 41

<210> SEQ ID NO 171
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 171

gggagttaac tccatcgcaa caacccacaa ccctgctagt g 41

<210> SEQ ID NO 172
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

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<400> SEQUENCE: 172

gtggtatcta tcgtgatgtg ac 22

<210> SEQ ID NO 173
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 173

ttaccgaaac ggaatttatc tgc 23

<210> SEQ ID NO 174
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 174

aaagctagag ttccgcaatt gg 22

<210> SEQ ID NO 175
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 175

gtgggttgta cggattgagt taactcccat ctccttc 37

<210> SEQ ID NO 176
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 176

gatgggagtt aactcaatcc gtacaaccca caaccctg 38

<210> SEQ ID NO 177
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 177

gcttcaccta ttgcagcacc aattgaccac atgaagatag 40

<210> SEQ ID NO 178
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

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<400> SEQUENCE: 178

gtggtcaatt ggtgctgcaa taggtgaagc taatggtgat g 41

<210> SEQ ID NO 179
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 179

ctgatttgta ttaattttga gacattatgc ttcaccttc 39

<210> SEQ ID NO 180
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 180

gcataatgtc tcaaaattaa tacaaatcag tgaaatcatg 40

<210> SEQ ID NO 181
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 181

gttttgggac cattcaaaac agcatagctc taaaacgtga cagtaatatc ag 52

<210> SEQ ID NO 182
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 182

gttttagagc tatgctgttt tgaatggtcc caaaacgctc actagcaggg ttg 53

<210> SEQ ID NO 183
<211> LENGTH: 59
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 183

atactttacg cagcgcggag ttcggttttg taggagtggt agtatataca cgagtacat 59

<210> SEQ ID NO 184
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

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oligonucleotide

<400> SEQUENCE: 184

gctcactagc agggttgtgg gttgtacgga tgg 33

<210> SEQ ID NO 185
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 185

tcctagcagg atttctgata ttactgtcac tgg 33

<210> SEQ ID NO 186
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 186

tttaaaagaa accgataccg tttacgaaat tgg 33

<210> SEQ ID NO 187
<211> LENGTH: 84
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 187

ggaaccattc ataacagcat agcaagttat aataaggcta gtccgttatc aacttgaaaa 60

agtggcaccg agtcggtgct tttt 84

<210> SEQ ID NO 188
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 188

gttatagagc tatgctgtta tgaatggtcc caaaac 36

<210> SEQ ID NO 189
<211> LENGTH: 84
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 189

ggaaccattc aatacagcat agcaagttaa tataaggcta gtccgttatc aacttgaaaa 60

agtggcaccg agtcggtgct tttt 84

<210> SEQ ID NO 190

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<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 190

gtattagagc tatgctgtat tgaatggtcc caaaac 36

<210> SEQ ID NO 191
<211> LENGTH: 103
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 191

nnnnnnnnnn nnnnnnnnnn gttttagagc tagaaatagc aagttaaaat aaggctagtc 60

cgttatcaac ttgaaaaagt ggcaccgagt cggtgctttt ttt 103

<210> SEQ ID NO 192
<211> LENGTH: 103
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 192

nnnnnnnnnn nnnnnnnnnn gtattagagc tagaaatagc aagttaatat aaggctagtc 60

cgttatcaac ttgaaaaagt ggcaccgagt cggtgctttt ttt 103

<210> SEQ ID NO 193
<211> LENGTH: 123
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 193

nnnnnnnnnn nnnnnnnnnn gttttagagc tatgctgttt tggaaacaaa acagcatagc 60

aagttaaaat aaggctagtc cgttatcaac ttgaaaaagt ggcaccgagt cggtgctttt 120

ttt 123

<210> SEQ ID NO 194
<211> LENGTH: 123
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

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polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 194

nnnnnnnnnn nnnnnnnnnn gtattagagc tatgctgtat tggaaacaat acagcatagc 60

aagttaatat aaggctagtc cgttatcaac ttgaaaaagt ggcaccgagt cggtgctttt 120

ttt 123

<210> SEQ ID NO 195
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 195

gtcacctcca atgactaggg 20

<210> SEQ ID NO 196
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 196

gacatcgatg tcctccccat tgg 23

<210> SEQ ID NO 197
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 197

gagtccgagc agaagaagaa ggg 23

<210> SEQ ID NO 198
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 198

gcgccaccgg ttgatgtgat ggg 23

<210> SEQ ID NO 199
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 199

ggggcacaga tgagaaactc agg 23

<210> SEQ ID NO 200
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 200

gtacaaacgg cagaagctgg agg 23

<210> SEQ ID NO 201
<211> LENGTH: 23

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 201

ggcagaagct ggaggaggaa ggg 23

<210> SEQ ID NO 202
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 202

ggagcccttc ttcttctgct cgg 23

<210> SEQ ID NO 203
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 203

gggcaaccac aaacccacga ggg 23

<210> SEQ ID NO 204
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 204

gctcccatca catcaaccgg tgg 23

<210> SEQ ID NO 205
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 205

gtggcgcatt gccacgaagc agg 23

<210> SEQ ID NO 206
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 206

ggcagagtgc tgcttgctgc tgg 23

<210> SEQ ID NO 207
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 207

gcccctgcgt gggcccaagc tgg 23

<210> SEQ ID NO 208
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 208

gagtggccag agtccagctt ggg 23

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<210> SEQ ID NO 209
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 209

ggcctcccca aagcctggcc agg 23

<210> SEQ ID NO 210
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 210

ggggccgaga ttgggtgttc agg 23

<210> SEQ ID NO 211
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 211

gtggcgagag gggccgagat tgg 23

<210> SEQ ID NO 212
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 212

gagtgccgcc gaggcggggc ggg 23

<210> SEQ ID NO 213
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 213

ggagtgccgc cgaggcgggg cgg 23

<210> SEQ ID NO 214
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 214

ggagaggagt gccgccgagg cgg 23

<210> SEQ ID NO 215
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 215

ccatcccctt ctgtgaatgt 20

<210> SEQ ID NO 216
<211> LENGTH: 20
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 216

ggagattgga gacacggaga 20

<210> SEQ ID NO 217
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 217

aagcaccgac tcggtgccac 20

<210> SEQ ID NO 218
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 218

tcacctccaa tgactagggg 20

<210> SEQ ID NO 219
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 219

caagttgata acggactagc ct 22

<210> SEQ ID NO 220
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 220

agtccgagca gaagaagaag ttt 23

<210> SEQ ID NO 221
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 221

tttcaagttg ataacggact agcct 25

<210> SEQ ID NO 222
<211> LENGTH: 20

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 222

aaacagcaga ttcgcctgga 20

<210> SEQ ID NO 223
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 223

tcatccgctc gatgaagctc 20

<210> SEQ ID NO 224
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 224

tccaaaatca agtggggcga 20

<210> SEQ ID NO 225
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 225

tgatgaccct tttggctccc 20

<210> SEQ ID NO 226
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 226

gaggaattct ttttttgtty gaatatgttg gaggtttttt ggaag 45

<210> SEQ ID NO 227
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 227

gagaagctta aataaaaaac racaatactc aacccaacaa cc 42

<210> SEQ ID NO 228

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<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 228

caggaaacag ctatgac 17

<210> SEQ ID NO 229
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 229

gcctctagag gtacctgagg gcctatttcc catgattcc 39

<210> SEQ ID NO 230
<211> LENGTH: 133
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (92)..(111)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 230

acctctagaa aaaaagcacc gactcggtgc cacttttttca agttgataac ggactagcct 60

tattttaact tgctatttct agctctaaaa cnnnnnnnnn nnnnnnnnnn nggtgtttcg 120

tcctttccac aag 133

<210> SEQ ID NO 231
<211> LENGTH: 133
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (92)..(111)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 231

acctctagaa aaaaagcacc gactcggtgc cacttttttca agttgataac ggactagcct 60

tatattaact tgctatttct agctctaata cnnnnnnnnn nnnnnnnnnn nggtgtttcg 120

tcctttccac aag 133

<210> SEQ ID NO 232
<211> LENGTH: 153
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (112)..(131)

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<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 232

acctctagaa aaaaagcacc gactcggtgc cacttttca agttgataac ggactagcct 60

tattttaact tgctatgctg ttttgtttcc aaaacagcat agctctaaaa cnnnnnnnnn 120

nnnnnnnnnn nggtgtttcg tcctttccac aag 153

<210> SEQ ID NO 233
<211> LENGTH: 153
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (112)..(131)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 233

acctctagaa aaaaagcacc gactcggtgc cacttttca agttgataac ggactagcct 60

tatattaact tgctatgctg tattgtttcc aatacagcat agctctaata cnnnnnnnnn 120

nnnnnnnnnn nggtgtttcg tcctttccac aag 153

<210> SEQ ID NO 234
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 234

aggccccagt ggctgctctn aa 22

<210> SEQ ID NO 235
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 235

acatcaaccg gtggcgcatn at 22

<210> SEQ ID NO 236
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 236

aaggtgtggt tccagaaccn ac 22

<210> SEQ ID NO 237
<211> LENGTH: 22
<212> TYPE: DNA

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<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 237

ccatcacatc aaccggtggn ag 22

<210> SEQ ID NO 238
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 238

aaacggcaga agctggaggn ta 22

<210> SEQ ID NO 239
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 239

ggcagaagct ggaggaggan tt 22

<210> SEQ ID NO 240
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 240

ggtgtggttc cagaaccggn tc 22

<210> SEQ ID NO 241
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 241

aaccggagga caaagtacan tg 22

<210> SEQ ID NO 242
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 242

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ttccagaacc ggaggacaan ca 22

<210> SEQ ID NO 243
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 243

gtgtggttcc agaaccggan ct 22

<210> SEQ ID NO 244
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 244

tccagaaccg gaggacaaan cc 22

<210> SEQ ID NO 245
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 245

cagaagctgg aggaggaagn cg 22

<210> SEQ ID NO 246
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 246

catcaaccgg tggcgcattn ga 22

<210> SEQ ID NO 247
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 247

gcagaagctg gaggaggaan gt 22

<210> SEQ ID NO 248
<211> LENGTH: 22
<212> TYPE: DNA

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<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 248

cctccctccc tggcccaggn gc 22

<210> SEQ ID NO 249
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 249

tcatctgtgc ccctccctcn aa 22

<210> SEQ ID NO 250
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 250

gggaggacat cgatgtcacn at 22

<210> SEQ ID NO 251
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 251

caaacggcag aagctggagn ac 22

<210> SEQ ID NO 252
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 252

gggtgggcaa ccacaaaccn ag 22

<210> SEQ ID NO 253
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 253

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ggtgggcaac cacaaacccn ta 22

<210> SEQ ID NO 254
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 254

ggctcccatc acatcaaccn tt 22

<210> SEQ ID NO 255
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 255

gaagggcctg agtccgagcn tc 22

<210> SEQ ID NO 256
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 256

caaccggtgg cgcattgccn tg 22

<210> SEQ ID NO 257
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 257

aggaggaagg gcctgagtcn ca 22

<210> SEQ ID NO 258
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 258

agctggagga ggaagggccn ct 22

<210> SEQ ID NO 259
<211> LENGTH: 22
<212> TYPE: DNA

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<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 259

gcattgccac gaagcaggcn cc 22

<210> SEQ ID NO 260
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 260

attgccacga agcaggccan cg 22

<210> SEQ ID NO 261
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 261

agaaccggag gacaaagtan ga 22

<210> SEQ ID NO 262
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 262

tcaaccggtg gcgcattgcn gt 22

<210> SEQ ID NO 263
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 263

gaagctggag gaggaagggn gc 22

<210> SEQ ID NO 264
<211> LENGTH: 123
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
polynucleotide

<400> SEQUENCE: 264

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ccaatgggga ggacatcgat gtcacctcca atgactaggg tgggcaacca caaacccacg 60

agggcagagt gctgcttgct gctggccagg cccctgcgtg ggcccaagct ggactctggc 120

cac 123

<210> SEQ ID NO 265
<211> LENGTH: 121
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 265

cgagcagaag aagaagggct cccatcacat caaccggtgg cgcattgcca cgaagcaggc 60

caatggggag gacatcgatg tcacctccaa tgactagggt gggcaaccac aaacccacga 120

g 121

<210> SEQ ID NO 266
<211> LENGTH: 128
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 266

ggaggacaaa gtacaaacgg cagaagctgg aggaggaagg gcctgagtcc gagcagaaga 60

agaagggctc ccatcacatc aaccggtggc gcattgccac gaagcaggcc aatggggagg 120

acatcgat 128

<210> SEQ ID NO 267
<211> LENGTH: 130
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 267

agaagctgga ggaggaaggg cctgagtccg agcagaagaa gaagggctcc catcacatca 60

accggtggcg cattgccacg aagcaggcca atggggagga catcgatgtc acctccaatg 120

actagggtgg 130

<210> SEQ ID NO 268
<211> LENGTH: 125
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 268

cctcagtctt cccatcaggc tctcagctca gcctgagtgt tgaggcccca gtggctgctc 60

tgggggcctc ctgagtttct catctgtgcc cctccctccc tggcccaggt gaaggtgtgg 120

ttcca 125

<210> SEQ ID NO 269
<211> LENGTH: 129

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 269

tcatctgtgc ccctccctcc ctggcccagg tgaaggtgtg gttccagaac cggaggacaa 60

agtacaaacg gcagaagctg gaggaggaag ggcctgagtc cgagcagaag aagaagggct 120

cccatcaca 129

<210> SEQ ID NO 270
<211> LENGTH: 129
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 270

ctccaatgac tagggtgggc aaccacaaac ccacgagggc agagtgctgc ttgctgctgg 60

ccaggcccct gcgtgggccc aagctggact ctggccactc cctggccagg ctttggggag 120

gcctggagt 129

<210> SEQ ID NO 271
<211> LENGTH: 127
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 271

ctgcttgctg ctggccaggc ccctgcgtgg gcccaagctg gactctggcc actccctggc 60

caggctttgg ggaggcctgg agtcatggcc ccacagggct tgaagcccgg ggccgccatt 120

gacagag 127

<210> SEQ ID NO 272
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 272

gaaattaata cgactcacta taggg 25

<210> SEQ ID NO 273
<211> LENGTH: 126
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 273

aaaaaagcac cgactcggtg ccacttttttc aagttgataa cggactagcc ttatttaac 60

ttgctatttc tagctctaaa acaacgacga gcgtgacacc accctatagt gagtcgtatt 120

aatttc 126

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<210> SEQ ID NO 274
<211> LENGTH: 126
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 274

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<210> SEQ ID NO 275
<211> LENGTH: 4677
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 275

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cgctccgatg ccgctccagg gcgagcgctg tttaaatagc caggcccccg attgcaaaga 180

cattatagcg agctaccaaa gccatattca aacacctaga tcactaccac ttctacacag 240

gccactcgag cttgtgatcg cactccgcta agggggcgcc tcttcctctt cgtttcagtc 300

acaacccgca aacatgtacc catacgatgt tccagattac gcttcgccga agaaaaagcg 360

caaggtcgaa gcgtccgaca agaagtacag catcggcctg gacatcggca ccaactctgt 420

gggctgggcc gtgatcaccg acgagtacaa ggtgcccagc aagaaattca aggtgctggg 480

caacaccgac cggcacagca tcaagaagaa cctgatcgga gccctgctgt tcgacagcgg 540

cgaaacagcc gaggccaccc ggctgaagag aaccgccaga agaagataca ccagacggaa 600

gaaccggatc tgctatctgc aagagatctt cagcaacgag atggccaagg tggacgacag 660

cttcttccac agactggaag agtccttcct ggtggaagag gataagaagc acgagcggca 720

ccccatcttc ggcaacatcg tggacgaggt ggcctaccac gagaagtacc ccaccatcta 780

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<210> SEQ ID NO 276
<211> LENGTH: 3150
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 276

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cgctccgatg ccgctccagg gcgagcgctg tttaaatagc caggcccccg attgcaaaga 180

cattatagcg agctaccaaa gccatattca aacacctaga tcactaccac ttctacacag 240

gccactcgag cttgtgatcg cactccgcta agggggcgcc tcttcctctt cgtttcagtc 300

acaacccgca aacatgccta agaagaagag gaaggttaac acgattaaca tcgctaagaa 360

cgacttctct gacatcgaac tggctgctat cccgttcaac actctggctg accattacgg 420

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caagccggaa gccgtagcgt acatcaccat taagaccact ctggcttgcc taaccagtgc 720

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<210> SEQ ID NO 277
<211> LENGTH: 125
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (23)..(42)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 277

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<210> SEQ ID NO 278
<211> LENGTH: 8452
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 278

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cttcacctag atccttttaa attaaaaatg aagttttaaa tcaatctaaa gtatatatga 180

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agttaatagt ttgcgcaacg ttgttgccat tgctacaggc atcgtggtgt cacgctcgtc 540

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cagacctaca accagctgtt cgaggaaaac cccatcaacg ccagcggcgt ggacgccaag 3300

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acctacgacg acgacctgga caacctgctg gcccagatcg gcgaccagta cgccgacctg 3540

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gaaaagggca agtccaagaa actgaagagt gtgaaagagc tgctggggat caccatcatg 6180

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gaaagaagca gcttcgagaa gaatcccatc gactttctgg aagccaaggg ctacaaagaa 6240

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<210> SEQ ID NO 279
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 279

gttttagagc tatgctgttt tgaatggtcc caaaacggaa gggcctgagt ccgagcagaa 60

gaagaagttt tagagctatg ctgttttgaa tggtcccaaa ac 102

<210> SEQ ID NO 280
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 280

cggaggacaa agtacaaacg gcagaagctg gaggaggaag ggcctgagtc cgagcagaag 60

aagaagggct cccatcacat caaccggtgg cgcattgcca 100

<210> SEQ ID NO 281
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 281

agctggagga ggaagggcct gagtccgagc agaagaagaa gggctcccac 50

<210> SEQ ID NO 282
<211> LENGTH: 30
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 282

gaguccgagc agaagaagaa guuuuagagc 30

<210> SEQ ID NO 283
<211> LENGTH: 49
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 283

agctggagga ggaagggcct gagtccgagc agaagagaag ggctcccat 49

<210> SEQ ID NO 284
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 284

ctggaggagg aagggcctga gtccgagcag aagaagaagg gctcccatca cat 53

<210> SEQ ID NO 285
<211> LENGTH: 52
<212> TYPE: DNA

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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 285

ctggaggagg aagggcctga gtccgagcag aagagaaggg ctcccatcac at 52

<210> SEQ ID NO 286
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 286

ctggaggagg aagggcctga gtccgagcag aagaaagaag ggctcccatc acat 54

<210> SEQ ID NO 287
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 287

ctggaggagg aagggcctga gtccgagcag aagaagggct cccatcacat 50

<210> SEQ ID NO 288
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 288

ctggaggagg aagggcctga gcccgagcag aagggctccc atcacat 47

<210> SEQ ID NO 289
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 289

nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaauagc aaguuaaaau aaggctagtc 60

cguuuu 66

<210> SEQ ID NO 290
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 290

gaguccgagc agaagaagaa 20

<210> SEQ ID NO 291
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 291

gacaucgaug uccuccccau 20

<210> SEQ ID NO 292
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 292

gucaccucca augacuaggg 20

<210> SEQ ID NO 293
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 293

auuggguguu cagggcagag 20

<210> SEQ ID NO 294
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 294

guggcgagag gggccgagau 20

<210> SEQ ID NO 295
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 295

ggggccgaga uuggguguuc 20

<210> SEQ ID NO 296
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 296

gugccauuag cuaaaugcau 20

<210> SEQ ID NO 297
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 297

guaccaccca caggugccag 20

<210> SEQ ID NO 298
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 298

gaaagccucu gggccaggaa 20

<210> SEQ ID NO 299
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 299

ctggaggagg aagggcctga gtccgagcag aagaagaagg gctcccat 48

<210> SEQ ID NO 300
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 300

gaguccgagc agaagaagau 20

<210> SEQ ID NO 301
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 301

gaguccgagc agaagaagua 20

<210> SEQ ID NO 302
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 302

gaguccgagc agaagaacaa 20

<210> SEQ ID NO 303
<211> LENGTH: 20
<212> TYPE: RNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 303

gaguccgagc agaagaugaa 20

<210> SEQ ID NO 304
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 304

gaguccgagc agaaguagaa 20

<210> SEQ ID NO 305
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 305

gaguccgagc agaugaagaa 20

<210> SEQ ID NO 306
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 306

gaguccgagc acaagaagaa 20

<210> SEQ ID NO 307
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 307

gaguccgagg agaagaagaa 20

<210> SEQ ID NO 308
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 308

gaguccgugc agaagaagaa 20

<210> SEQ ID NO 309
<211> LENGTH: 20

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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 309

gagucggagc agaagaagaa 20

<210> SEQ ID NO 310
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 310

gagaccgagc agaagaagaa 20

<210> SEQ ID NO 311
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 311

aatgacaagc ttgctagcgg tggg 24

<210> SEQ ID NO 312
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 312

aaaacggaag ggcctgagtc cgagcagaag aagaagttt 39

<210> SEQ ID NO 313
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 313

aaacaggggc cgagattggg tgttcagggc agaggtttt 39

<210> SEQ ID NO 314
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 314

aaaacggaag ggcctgagtc cgagcagaag aagaagtt 38

<210> SEQ ID NO 315

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<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 315

aacggaggga ggggcacaga tgagaaactc agggttttag 40

<210> SEQ ID NO 316
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 316

agcccttctt cttctgctcg gactcaggcc cttcctcc 38

<210> SEQ ID NO 317
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 317

cagggaggga ggggcacaga tgagaaactc aggaggcccc 40

<210> SEQ ID NO 318
<211> LENGTH: 80
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 318

ggcaatgcgc caccggttga tgtgatggga gcccttctag gaggccccca gagcagccac 60

tggggcctca acactcaggc 80

<210> SEQ ID NO 319
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 319

gtcacctcca atgactaggg tgg 23

<210> SEQ ID NO 320
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (6)..(25)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 320

caccgnnnnn nnnnnnnnnn nnnnn 25

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<210> SEQ ID NO 321
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (5)..(24)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 321

aaacnnnnnn nnnnnnnnnn nnnnc 25

<210> SEQ ID NO 322
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 322

catcgatgtc ctccccattg gcctgcttcg tgg 33

<210> SEQ ID NO 323
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 323

ttcgtggcaa tgcgccaccg gttgatgtga tgg 33

<210> SEQ ID NO 324
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 324

tcgtggcaat gcgccaccgg ttgatgtgat ggg 33

<210> SEQ ID NO 325
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 325

tccagcttct gccgtttgta ctttgtcctc cgg 33

<210> SEQ ID NO 326
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 326

ggagggaggg gcacagatga gaaactcagg agg 33

<210> SEQ ID NO 327
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 327

aggggccgag attgggtgtt cagggcagag agg 33

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<210> SEQ ID NO 328
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 328

aacaccgggt cttcgagaag acctgtttta gagctagaaa tagcaagtta aaat 54

<210> SEQ ID NO 329
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 329

caaaacgggt cttcgagaag acgttttaga gctatgctgt tttgaatggt ccca 54

<210> SEQ ID NO 330
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 330

caagcactga gtgccattag ctaaatgcat agg 33

<210> SEQ ID NO 331
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 331

aatgcatagg gtaccaccca caggtgccag ggg 33

<210> SEQ ID NO 332
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 332

acacacatgg gaaagcctct gggccaggaa agg 33

<210> SEQ ID NO 333
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 333

ggaggaggta gtatacagaa acacagagaa gtagaat 37

<210> SEQ ID NO 334
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 334

agaatgtaga ggagtcacag aaactcagca ctagaaa 37

<210> SEQ ID NO 335

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<211> LENGTH: 98
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 335

ggacgaaaca ccggaaccat tcaaaacagc atagcaagtt aaaataaggc tagtccgtta 60

tcaacttgaa aaagtggcac cgagtcggtg ctttttttt 98

<210> SEQ ID NO 336
<211> LENGTH: 186
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 336

ggacgaaaca ccggtagtat taagtattgt tttatggctg ataaatttct ttgaatttct 60

ccttgattat ttgttataaa agttataaaa taatcttgtt ggaaccattc aaaacagcat 120

agcaagttaa aataaggcta gtccgttatc aacttgaaaa agtggcaccg agtcggtgct 180

tttttt 186

<210> SEQ ID NO 337
<211> LENGTH: 95
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 337

gggttttaga gctatgctgt tttgaatggt cccaaaacgg gtcttcgaga agacgtttta 60

gagctatgct gttttgaatg gtcccaaaac ttttt 95

<210> SEQ ID NO 338
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (5)..(34)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 338

aaacnnnnnn nnnnnnnnnn nnnnnnnnnn nnnngt 36

<210> SEQ ID NO 339
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (7)..(36)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

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<400> SEQUENCE: 339

taaaacnnnn nnnnnnnnnn nnnnnnnnnn nnnnnn 36

<210> SEQ ID NO 340
<211> LENGTH: 84
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 340

gtggaaagga cgaaacaccg ggtcttcgag aagacctgtt ttagagctag aaatagcaag 60

ttaaaataag gctagtccgt tttt 84

<210> SEQ ID NO 341
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (6)..(24)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 341

caccgnnnnn nnnnnnnnnn nnnn 24

<210> SEQ ID NO 342
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (5)..(23)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 342

aaacnnnnnn nnnnnnnnnn nnnc 24

<210> SEQ ID NO 343
<211> LENGTH: 88
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 343

gttttagagc tatgctgttt tgaatggtcc caaaactgag accaaaggtc tcgttttaga 60

gctatgctgt tttgaatggt cccaaaac 88

<210> SEQ ID NO 344
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

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<400> SEQUENCE: 344

aaacggaagg gcctgagtcc gagcagaaga agaag 35

<210> SEQ ID NO 345
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 345

aaaacttctt cttctgctcg gactcaggcc cttcc 35

<210> SEQ ID NO 346
<211> LENGTH: 46
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(19)
<223> OTHER INFORMATION: a, c, u, g, unknown or other

<400> SEQUENCE: 346

nnnnnnnnnn nnnnnnnnng uuauuguacu cucaagauuu auuuuu 46

<210> SEQ ID NO 347
<211> LENGTH: 91
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 347

guuacuuaaa ucuugcagaa gcuacaaaga uaaggcuuca ugccgaaauc aacacccugu 60

cauuuuaugg caggguguuu ucguuauuua a 91

<210> SEQ ID NO 348
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 348

ttttctagtg ctgagtttct gtgactcctc tacattctac ttctctgtgt ttctgtatac 60

tacctcctcc 70

<210> SEQ ID NO 349
<211> LENGTH: 122
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 349

ggaggaaggg cctgagtccg agcagaagaa gaagggctcc catcacatca accggtggcg 60

cattgccacg aagcaggcca atggggagga catcgatgtc acctccaatg actagggtgg 120

gc 122

<210> SEQ ID NO 350

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<211> LENGTH: 48
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (3)..(32)
<223> OTHER INFORMATION: a, c, u, g, unknown or other

<400> SEQUENCE: 350

acnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnguuuuaga gcuaugcu 48

<210> SEQ ID NO 351
<211> LENGTH: 67
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic oligonucleotide

<400> SEQUENCE: 351

agcauagcaa guuaaaauaa ggctaguccg uuaucaacuu gaaaaagugg caccgagucg 60

gugcuuu 67

<210> SEQ ID NO 352
<211> LENGTH: 62
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, u, g, unknown or other

<400> SEQUENCE: 352

nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cg 62

<210> SEQ ID NO 353
<211> LENGTH: 73
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 353

tgaatggtcc caaaacggaa gggcctgagt ccgagcagaa gaagaagttt tagagctatg 60

ctgttttgaa tgg 73

<210> SEQ ID NO 354
<211> LENGTH: 69
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 354

ctggtcttcc acctctctgc cctgaacacc caatctcggc ccctctcgcc accctcctgc 60

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atttctgtt 69

<210> SEQ ID NO 355
<211> LENGTH: 138
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 355

acccaagcac tgagtgccat tagctaaatg catagggtac cacccacagg tgccaggggc 60

ctttcccaaa gttcccagcc ccttctccaa cctttcctgg cccagaggct ttcccatgtg 120

tgtggctgga ccctttga 138

<210> SEQ ID NO 356
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 356

gtgctttgca gaggcctacc 20

<210> SEQ ID NO 357
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 357

cctggagcgc atgcagtagt 20

<210> SEQ ID NO 358
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 358

accttctgtg tttccaccat tc 22

<210> SEQ ID NO 359
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 359

ttggggagtg cacagacttc 20

<210> SEQ ID NO 360
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

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<400> SEQUENCE: 360

ggctccctgg gttcaaagta 20

<210> SEQ ID NO 361
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 361

agaggggtct ggatgtcgta a 21

<210> SEQ ID NO 362
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic probe

<400> SEQUENCE: 362

tagctctaaa acttcttctt ctgctcggac 30

<210> SEQ ID NO 363
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic probe

<400> SEQUENCE: 363

ctagccttat tttaacttgc tatgctgttt 30

<210> SEQ ID NO 364
<211> LENGTH: 99
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, u, g, unknown or other

<400> SEQUENCE: 364

nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuu 99

<210> SEQ ID NO 365
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 365

tagcgggtaa gc 12

<210> SEQ ID NO 366
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 366

tcggtgacat gt 12

<210> SEQ ID NO 367
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 367

actccccgta gg 12

<210> SEQ ID NO 368
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 368

actgcgtgtt aa 12

<210> SEQ ID NO 369
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 369

acgtcgcctg at 12

<210> SEQ ID NO 370
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 370

taggtcgacc ag 12

<210> SEQ ID NO 371
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 371

ggcgttaatg at 12

<210> SEQ ID NO 372
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 372

tgtcgcatgt ta 12

<210> SEQ ID NO 373
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 373

atggaaacgc at 12

<210> SEQ ID NO 374

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<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 374

gccgaattcc tc 12

<210> SEQ ID NO 375
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 375

gcatggtacg ga 12

<210> SEQ ID NO 376
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 376

cggtactctt ac 12

<210> SEQ ID NO 377
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 377

gcctgtgccg ta 12

<210> SEQ ID NO 378
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 378

tacggtaagt cg 12

<210> SEQ ID NO 379
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 379

cacgaaatta cc 12

<210> SEQ ID NO 380
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 380

aaccaagata cg 12

<210> SEQ ID NO 381
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 381

gagtcgatac gc 12

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<210> SEQ ID NO 382
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 382

gtctcacgat cg 12

<210> SEQ ID NO 383
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 383

tcgtcgggtg ca 12

<210> SEQ ID NO 384
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 384

actccgtagt ga 12

<210> SEQ ID NO 385
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 385

caggacgtcc gt 12

<210> SEQ ID NO 386
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 386

tcgtatccct ac 12

<210> SEQ ID NO 387
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 387

tttcaaggcc gg 12

<210> SEQ ID NO 388
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 388

cgccggtgga at 12

<210> SEQ ID NO 389
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 389

gaacccgtcc ta 12

<210> SEQ ID NO 390
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 390

gattcatcag cg 12

<210> SEQ ID NO 391
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 391

acaccggtct tc 12

<210> SEQ ID NO 392
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 392

atcgtgccct aa 12

<210> SEQ ID NO 393
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 393

gcgtcaatgt tc 12

<210> SEQ ID NO 394
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 394

ctccgtatct cg 12

<210> SEQ ID NO 395
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 395

ccgattcctt cg 12

<210> SEQ ID NO 396
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 396

tgcgcctcca gt 12

<210> SEQ ID NO 397
<211> LENGTH: 12

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 397

taacgtcgga gc 12

<210> SEQ ID NO 398
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 398

aaggtcgccc at 12

<210> SEQ ID NO 399
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 399

gtcggggact at 12

<210> SEQ ID NO 400
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 400

ttcgagcgat tt 12

<210> SEQ ID NO 401
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 401

tgagtcgtcg ag 12

<210> SEQ ID NO 402
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 402

tttacgcaga gg 12

<210> SEQ ID NO 403
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 403

aggaagtatc gc 12

<210> SEQ ID NO 404
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 404

actcgatacc at 12

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<210> SEQ ID NO 405
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 405

cgctacatag ca 12

<210> SEQ ID NO 406
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 406

ttcataaccg gc 12

<210> SEQ ID NO 407
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 407

ccaaacggtt aa 12

<210> SEQ ID NO 408
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 408

cgattccttc gt 12

<210> SEQ ID NO 409
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 409

cgtcatgaat aa 12

<210> SEQ ID NO 410
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 410

agtggcgatg ac 12

<210> SEQ ID NO 411
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 411

cccctacggc ac 12

<210> SEQ ID NO 412
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 412

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gccaacccgc ac 12

<210> SEQ ID NO 413
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 413

tgggacaccg gt 12

<210> SEQ ID NO 414
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 414

ttgactgcgg cg 12

<210> SEQ ID NO 415
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 415

actatgcgta gg 12

<210> SEQ ID NO 416
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 416

tcacccaaag cg 12

<210> SEQ ID NO 417
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 417

gcaggacgtc cg 12

<210> SEQ ID NO 418
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 418

acaccgaaaa cg 12

<210> SEQ ID NO 419
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 419

cggtgtattg ag 12

<210> SEQ ID NO 420
<211> LENGTH: 12
<212> TYPE: DNA

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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 420

cacgaggtat gc 12

<210> SEQ ID NO 421
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 421

taaagcgacc cg 12

<210> SEQ ID NO 422
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 422

cttagtcggc ca 12

<210> SEQ ID NO 423
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 423

cgaaaacgtg gc 12

<210> SEQ ID NO 424
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 424

cgtgccctga ac 12

<210> SEQ ID NO 425
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 425

tttaccatcg aa 12

<210> SEQ ID NO 426
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 426

cgtagccatg tt 12

<210> SEQ ID NO 427
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 427

cccaaacggt ta 12

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<210> SEQ ID NO 428
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 428

gcgttatcag aa 12

<210> SEQ ID NO 429
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 429

tcgatggtaa ac 12

<210> SEQ ID NO 430
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 430

cgacttttg ca 12

<210> SEQ ID NO 431
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 431

tcgacgactc ac 12

<210> SEQ ID NO 432
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 432

acgcgtcaga ta 12

<210> SEQ ID NO 433
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 433

cgtacggcac ag 12

<210> SEQ ID NO 434
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 434

ctatgccgtg ca 12

<210> SEQ ID NO 435
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 435

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cgcgtcagat at 12

<210> SEQ ID NO 436
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 436

aagatcggta gc 12

<210> SEQ ID NO 437
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 437

cttcgcaagg ag 12

<210> SEQ ID NO 438
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 438

gtcgtggact ac 12

<210> SEQ ID NO 439
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 439

ggtcgtcatc aa 12

<210> SEQ ID NO 440
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 440

gttaacagcg tg 12

<210> SEQ ID NO 441
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 441

tagctaaccg tt 12

<210> SEQ ID NO 442
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 442

agtaaaggcg ct 12

<210> SEQ ID NO 443
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 443

ggtaatttcg tg 12

<210> SEQ ID NO 444
<211> LENGTH: 69
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 444

gucaccucca augacuaggg guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuuuuuu 69

<210> SEQ ID NO 445
<211> LENGTH: 69
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 445

gacaucgaug uccuccccau guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuuuuuu 69

<210> SEQ ID NO 446
<211> LENGTH: 69
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 446

gaguccgagc agaagaagaa guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuuuuuu 69

<210> SEQ ID NO 447
<211> LENGTH: 69
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 447

ggggccgaga uuggguguuc guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuuuuuu 69

<210> SEQ ID NO 448
<211> LENGTH: 69
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 448

guggcgagag gggccgagau guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

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cguuuuuuu 69

<210> SEQ ID NO 449
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 449

gucaccucca augacuaggg guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucauu uuuuuu 76

<210> SEQ ID NO 450
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 450

gacaucgaug uccucccccau guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucauu uuuuuu 76

<210> SEQ ID NO 451
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 451

gaguccgagc agaagaagaa guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucauu uuuuuu 76

<210> SEQ ID NO 452
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 452

ggggccgaga uuggguguuc guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucauu uuuuuu 76

<210> SEQ ID NO 453
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 453

guggcgagag gggccgagau guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucauu uuuuuu 76

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<210> SEQ ID NO 454
<211> LENGTH: 88
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 454

gucaccucca augacuaggg guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu guuuuuuu 88

<210> SEQ ID NO 455
<211> LENGTH: 88
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 455

gacaucgaug uccuccccau guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu guuuuuuu 88

<210> SEQ ID NO 456
<211> LENGTH: 88
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 456

gaguccgagc agaagaagaa guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu guuuuuuu 88

<210> SEQ ID NO 457
<211> LENGTH: 88
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 457

ggggccgaga uuggguguuc guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu guuuuuuu 88

<210> SEQ ID NO 458
<211> LENGTH: 88
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 458

guggcgagag gggccgagau guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu guuuuuuu 88

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<210> SEQ ID NO 459
<211> LENGTH: 103
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 459

gucaccucca augacuaggg guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu uuu 103

<210> SEQ ID NO 460
<211> LENGTH: 103
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 460

gacaucgaug uccuccccau guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu uuu 103

<210> SEQ ID NO 461
<211> LENGTH: 103
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 461

gaguccgagc agaagaagaa guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu uuu 103

<210> SEQ ID NO 462
<211> LENGTH: 103
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 462

ggggccgaga uuggguguuc guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu uuu 103

<210> SEQ ID NO 463
<211> LENGTH: 103
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 463

guggcgagag gggccgagau guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu uuu 103

<210> SEQ ID NO 464
<211> LENGTH: 120

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 464

gtggaaagga cgaaacaccg ggtcttcgag aagacctgtt ttagagctag aaatagcaag 60

ttaaaataag gctagtccgt tatcaacttg aaaaagtggc accgagtcgg tgctttttt 120

<210> SEQ ID NO 465
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 465

tcggtgcgct ggttgatttc ttcttgcgct tttttggctt 40

<210> SEQ ID NO 466
<211> LENGTH: 26
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 466

gauuucuucu ugcgcuuuuu guuuua 26

<210> SEQ ID NO 467
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (22)..(26)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 467

tgatttcttc ttgcgctttt tnnnnn 26

<210> SEQ ID NO 468
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (21)..(21)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 468

tgatttcttc ttgcgctttt ntggct 26

<210> SEQ ID NO 469
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 469

tnatttcttc ttgcgctttt ttggct 26

<210> SEQ ID NO 470
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 470

gatttcttct tgcgcttttt tgg 23

<210> SEQ ID NO 471
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(33)

<400> SEQUENCE: 471

tcc atc cgt aca acc cac aac cct gct agt gag c 34
Ser Ile Arg Thr Thr His Asn Pro Ala Ser Glu
1 5 10

<210> SEQ ID NO 472
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 472

Ser Ile Arg Thr Thr His Asn Pro Ala Ser Glu
1 5 10

<210> SEQ ID NO 473
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(33)

<400> SEQUENCE: 473

tcc atc gca aca acc cac aac cct gct agt gag c 34
Ser Ile Ala Thr Thr His Asn Pro Ala Ser Glu
1 5 10

<210> SEQ ID NO 474

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<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 474

Ser Ile Ala Thr Thr His Asn Pro Ala Ser Glu
1 5 10

<210> SEQ ID NO 475
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(33)

<400> SEQUENCE: 475

tca atc cgt aca acc cac aac cct gct agt gag c 34
Ser Ile Arg Thr Thr His Asn Pro Ala Ser Glu
1 5 10

<210> SEQ ID NO 476
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(36)

<400> SEQUENCE: 476

caa ttg aat tta aaa gaa acc gat acc gtt ttg gtt taagga 42
Gln Leu Asn Leu Lys Glu Thr Asp Thr Val Leu Val
1 5 10

<210> SEQ ID NO 477
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 477

Gln Leu Asn Leu Lys Glu Thr Asp Thr Val Leu Val
1 5 10

<210> SEQ ID NO 478
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(42)

<400> SEQUENCE: 478

caa ttg aat tta aaa gaa acc gat acc gtt tac gaa att gga 42
Gln Leu Asn Leu Lys Glu Thr Asp Thr Val Tyr Glu Ile Gly
1 5 10

<210> SEQ ID NO 479
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 479

Gln Leu Asn Leu Lys Glu Thr Asp Thr Val Tyr Glu Ile Gly
1 5 10

<210> SEQ ID NO 480
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (2)..(34)

<400> SEQUENCE: 480

t cct aaa aaa ccg aac tcc gcg ctg cgt aaa gta 34
Pro Lys Lys Pro Asn Ser Ala Leu Arg Lys Val
1 5 10

<210> SEQ ID NO 481
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 481

Pro Lys Lys Pro Asn Ser Ala Leu Arg Lys Val
1 5 10

<210> SEQ ID NO 482
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (2)..(34)

<400> SEQUENCE: 482

t cct aca aaa ccg aac tcc gcg ctg cgt aaa gta 34
Pro Thr Lys Pro Asn Ser Ala Leu Arg Lys Val
1 5 10

<210> SEQ ID NO 483
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 483

Pro Thr Lys Pro Asn Ser Ala Leu Arg Lys Val
1 5 10

<210> SEQ ID NO 484
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 484

tgcgctggtt gatttcttct tgcgcttttt tgg 33

<210> SEQ ID NO 485
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 485

tacgctggtt gatttcttct tgcgcttttt ttg 33

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<210> SEQ ID NO 486
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 486

ggagggtttt atggggaaag gccattg 27

<210> SEQ ID NO 487
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 487

gtaaaaaaga agactagaaa ttttgatac 29

<210> SEQ ID NO 488
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 488

ggagggtttt atggggaaag gcaaagaaga ctagaaattt tgatac 46

<210> SEQ ID NO 489
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 489

aggtgaagca taatgtctca aaaaata 27

<210> SEQ ID NO 490
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 490

attttattaa tacaaatcag tgaaatcat 29

<210> SEQ ID NO 491
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 491

aggtgaagca taatgtctca aaattaatac aaatcagtga aatcat 46

<210> SEQ ID NO 492
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(36)

<400> SEQUENCE: 492

aat tta aaa gaa acc gat acc gtt tac gaa att gga 36
Asn Leu Lys Glu Thr Asp Thr Val Tyr Glu Ile Gly
1 5 10

<210> SEQ ID NO 493

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<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 493

Asn Leu Lys Glu Thr Asp Thr Val Tyr Glu Ile Gly
1 5 10

<210> SEQ ID NO 494
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(30)

<400> SEQUENCE: 494

aat tta aaa gaa acc gat acc gtt ttg gtt taagga 36
Asn Leu Lys Glu Thr Asp Thr Val Leu Val
1 5 10

<210> SEQ ID NO 495
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 495

Asn Leu Lys Glu Thr Asp Thr Val Leu Val
1 5 10

<210> SEQ ID NO 496
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(36)

<400> SEQUENCE: 496

tgg gat cca aaa aaa tat ggt ggt ttt gat agt cca 36
Trp Asp Pro Lys Lys Tyr Gly Gly Phe Asp Ser Pro
1 5 10

<210> SEQ ID NO 497
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 497

Trp Asp Pro Lys Lys Tyr Gly Gly Phe Asp Ser Pro
1 5 10

<210> SEQ ID NO 498
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(36)

<400> SEQUENCE: 498

tgg gat cca aaa aaa tat tgt ggt ttt gat agt cca 36
Trp Asp Pro Lys Lys Tyr Cys Gly Phe Asp Ser Pro

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1               5                   10


<210> SEQ ID NO 499
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      peptide

<400> SEQUENCE: 499

Trp Asp Pro Lys Lys Tyr Cys Gly Phe Asp Ser Pro
1               5                   10


<210> SEQ ID NO 500
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      oligonucleotide

<400> SEQUENCE: 500

aaactacttt acgcagcgcg gagttcggtt ttttg                                35


<210> SEQ ID NO 501
<211> LENGTH: 4104
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(4104)

<400> SEQUENCE: 501

atg gac aag aag tac agc atc ggc ctg gac atc ggc acc aac tct gtg       48
Met Asp Lys Lys Tyr Ser Ile Gly Leu Asp Ile Gly Thr Asn Ser Val
1               5                   10                  15

ggc tgg gcc gtg atc acc gac gag tac aag gtg ccc agc aag aaa ttc       96
Gly Trp Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe
            20                  25                  30

aag gtg ctg ggc aac acc gac cgg cac agc atc aag aag aac ctg atc      144
Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile
        35                  40                  45

gga gcc ctg ctg ttc gac agc ggc gaa aca gcc gag gcc acc cgg ctg      192
Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
    50                  55                  60

aag aga acc gcc aga aga aga tac acc aga cgg aag aac cgg atc tgc      240
Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
65                  70                  75                  80

tat ctg caa gag atc ttc agc aac gag atg gcc aag gtg gac gac agc      288
Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser
                85                  90                  95

ttc ttc cac aga ctg gaa gag tcc ttc ctg gtg gaa gag gat aag aag      336
Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
            100                 105                 110

cac gag cgg cac ccc atc ttc ggc aac atc gtg gac gag gtg gcc tac      384
His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr
        115                 120                 125

cac gag aag tac ccc acc atc tac cac ctg aga aag aaa ctg gtg gac      432
His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp
    130                 135                 140

agc acc gac aag gcc gac ctg cgg ctg atc tat ctg gcc ctg gcc cac      480
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Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
145                 150                 155                 160

atg atc aag ttc cgg ggc cac ttc ctg atc gag ggc gac ctg aac ccc      528
Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
                165                 170                 175

gac aac agc gac gtg gac aag ctg ttc atc cag ctg gtg cag acc tac      576
Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr
            180                 185                 190

aac cag ctg ttc gag gaa aac ccc atc aac gcc agc ggc gtg gac gcc      624
Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp Ala
        195                 200                 205

aag gcc atc ctg tct gcc aga ctg agc aag agc aga cgg ctg gaa aat      672
Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn
    210                 215                 220

ctg atc gcc cag ctg ccc ggc gag aag aag aat ggc ctg ttc ggc aac      720
Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn
225                 230                 235                 240

ctg att gcc ctg agc ctg ggc ctg acc ccc aac ttc aag agc aac ttc      768
Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe
                245                 250                 255

gac ctg gcc gag gat gcc aaa ctg cag ctg agc aag gac acc tac gac      816
Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp
            260                 265                 270

gac gac ctg gac aac ctg ctg gcc cag atc ggc gac cag tac gcc gac      864
Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp
        275                 280                 285

ctg ttt ctg gcc gcc aag aac ctg tcc gac gcc atc ctg ctg agc gac      912
Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp
    290                 295                 300

atc ctg aga gtg aac acc gag atc acc aag gcc ccc ctg agc gcc tct      960
Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser
305                 310                 315                 320

atg atc aag aga tac gac gag cac cac cag gac ctg acc ctg ctg aaa     1008
Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys
                325                 330                 335

gct ctc gtg cgg cag cag ctg cct gag aag tac aaa gag att ttc ttc     1056
Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe
            340                 345                 350

gac cag agc aag aac ggc tac gcc ggc tac att gac ggc gga gcc agc     1104
Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser
        355                 360                 365

cag gaa gag ttc tac aag ttc atc aag ccc atc ctg gaa aag atg gac     1152
Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp
    370                 375                 380

ggc acc gag gaa ctg ctc gtg aag ctg aac aga gag gac ctg ctg cgg     1200
Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg
385                 390                 395                 400

aag cag cgg acc ttc gac aac ggc agc atc ccc cac cag atc cac ctg     1248
Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu
                405                 410                 415

gga gag ctg cac gcc att ctg cgg cgg cag gaa gat ttt tac cca ttc     1296
Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe
            420                 425                 430

ctg aag gac aac cgg gaa aag atc gag aag atc ctg acc ttc cgc atc     1344
Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile
        435                 440                 445

ccc tac tac gtg ggc cct ctg gcc agg gga aac agc aga ttc gcc tgg     1392
Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp
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    450                 455                 460

atg acc aga aag agc gag gaa acc atc acc ccc tgg aac ttc gag gaa     1440
Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu
465                 470                 475                 480

gtg gtg gac aag ggc gct tcc gcc cag agc ttc atc gag cgg atg acc     1488
Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr
                485                 490                 495

aac ttc gat aag aac ctg ccc aac gag aag gtg ctg ccc aag cac agc     1536
Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser
            500                 505                 510

ctg ctg tac gag tac ttc acc gtg tat aac gag ctg acc aaa gtg aaa     1584
Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys
        515                 520                 525

tac gtg acc gag gga atg aga aag ccc gcc ttc ctg agc ggc gag cag     1632
Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln
    530                 535                 540

aaa aag gcc atc gtg gac ctg ctg ttc aag acc aac cgg aaa gtg acc     1680
Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr
545                 550                 555                 560

gtg aag cag ctg aaa gag gac tac ttc aag aaa atc gag tgc ttc gac     1728
Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp
                565                 570                 575

tcc gtg gaa atc tcc ggc gtg gaa gat cgg ttc aac gcc tcc ctg ggc     1776
Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly
            580                 585                 590

aca tac cac gat ctg ctg aaa att atc aag gac aag gac ttc ctg gac     1824
Thr Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp
        595                 600                 605

aat gag gaa aac gag gac att ctg gaa gat atc gtg ctg acc ctg aca     1872
Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr
    610                 615                 620

ctg ttt gag gac aga gag atg atc gag gaa cgg ctg aaa acc tat gcc     1920
Leu Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala
625                 630                 635                 640

cac ctg ttc gac gac aaa gtg atg aag cag ctg aag cgg cgg aga tac     1968
His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr
                645                 650                 655

acc ggc tgg ggc agg ctg agc cgg aag ctg atc aac ggc atc cgg gac     2016
Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp
            660                 665                 670

aag cag tcc ggc aag aca atc ctg gat ttc ctg aag tcc gac ggc ttc     2064
Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe
        675                 680                 685

gcc aac aga aac ttc atg cag ctg atc cac gac gac agc ctg acc ttt     2112
Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr Phe
    690                 695                 700

aaa gag gac atc cag aaa gcc cag gtg tcc ggc cag ggc gat agc ctg     2160
Lys Glu Asp Ile Gln Lys Ala Gln Val Ser Gly Gln Gly Asp Ser Leu
705                 710                 715                 720

cac gag cac att gcc aat ctg gcc ggc agc ccc gcc att aag aag ggc     2208
His Glu His Ile Ala Asn Leu Ala Gly Ser Pro Ala Ile Lys Lys Gly
                725                 730                 735

atc ctg cag aca gtg aag gtg gtg gac gag ctc gtg aaa gtg atg ggc     2256
Ile Leu Gln Thr Val Lys Val Val Asp Glu Leu Val Lys Val Met Gly
            740                 745                 750

cgg cac aag ccc gag aac atc gtg atc gcc atg gcc aga gag aac cag     2304
Arg His Lys Pro Glu Asn Ile Val Ile Ala Met Ala Arg Glu Asn Gln
        755                 760                 765
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acc acc cag aag gga cag aag aac agc cgc gag aga atg aag cgg atc     2352
Thr Thr Gln Lys Gly Gln Lys Asn Ser Arg Glu Arg Met Lys Arg Ile
    770                 775                 780

gaa gag ggc atc aaa gag ctg ggc agc cag atc ctg aaa gaa cac ccc     2400
Glu Glu Gly Ile Lys Glu Leu Gly Ser Gln Ile Leu Lys Glu His Pro
785                 790                 795                 800

gtg gaa aac acc cag ctg cag aac gag aag ctg tac ctg tac tac ctg     2448
Val Glu Asn Thr Gln Leu Gln Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu
                805                 810                 815

cag aat ggg cgg gat atg tac gtg gac cag gaa ctg gac atc aac cgg     2496
Gln Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg
            820                 825                 830

ctg tcc gac tac gat gtg gac gcc atc gtg cct cag agc ttt ctg aag     2544
Leu Ser Asp Tyr Asp Val Asp Ala Ile Val Pro Gln Ser Phe Leu Lys
        835                 840                 845

gac gac tcc atc gac gcc aag gtg ctg acc aga agc gac aag gcc cgg     2592
Asp Asp Ser Ile Asp Ala Lys Val Leu Thr Arg Ser Asp Lys Ala Arg
    850                 855                 860

ggc aag agc gac aac gtg ccc tcc gaa gag gtc gtg aag aag atg aag     2640
Gly Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys
865                 870                 875                 880

aac tac tgg cgg cag ctg ctg aac gcc aag ctg att acc cag aga aag     2688
Asn Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys
                885                 890                 895

ttc gac aat ctg acc aag gcc gag aga ggc ggc ctg agc gaa ctg gat     2736
Phe Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp
            900                 905                 910

aag gcc ggc ttc atc aag aga cag ctg gtg gaa acc cgg cag atc aca     2784
Lys Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr
        915                 920                 925

aag cac gtg gca cag atc ctg gac tcc cgg atg aac act aag tac gac     2832
Lys His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp
    930                 935                 940

gag aat gac aag ctg atc cgg gaa gtg aaa gtg atc acc ctg aag tcc     2880
Glu Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser
945                 950                 955                 960

aag ctg gtg tcc gat ttc cgg aag gat ttc cag ttt tac aaa gtg cgc     2928
Lys Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg
                965                 970                 975

gag atc aac aac tac cac cac gcc cac gcc gcc tac ctg aac gcc gtc     2976
Glu Ile Asn Asn Tyr His His Ala His Ala Ala Tyr Leu Asn Ala Val
            980                 985                 990

gtg gga acc gcc ctg atc aaa aag  tac cct aag ctg gaa  agc gag ttc   3024
Val Gly Thr Ala Leu Ile Lys Lys  Tyr Pro Lys Leu Glu  Ser Glu Phe
        995                 1000                1005

gtg tac  ggc gac tac aag gtg  tac gac gtg cgg aag  atg atc gcc      3069
Val Tyr  Gly Asp Tyr Lys Val  Tyr Asp Val Arg Lys  Met Ile Ala
    1010                1015                1020

aag agc  gag cag gaa atc ggc  aag gct acc gcc aag  tac ttc ttc      3114
Lys Ser  Glu Gln Glu Ile Gly  Lys Ala Thr Ala Lys  Tyr Phe Phe
    1025                1030                1035

tac agc  aac atc atg aac ttt  ttc aag acc gag att  acc ctg gcc      3159
Tyr Ser  Asn Ile Met Asn Phe  Phe Lys Thr Glu Ile  Thr Leu Ala
    1040                1045                1050

aac ggc  gag atc cgg aag cgg  cct ctg atc gag aca  aac ggc gaa      3204
Asn Gly  Glu Ile Arg Lys Arg  Pro Leu Ile Glu Thr  Asn Gly Glu
    1055                1060                1065
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acc ggg  gag atc gtg tgg gat  aag ggc cgg gat ttt  gcc acc gtg      3249
Thr Gly  Glu Ile Val Trp Asp  Lys Gly Arg Asp Phe  Ala Thr Val
    1070                1075                1080

cgg aaa  gtg ctg agc atg ccc  caa gtg aat atc gtg  aaa aag acc      3294
Arg Lys  Val Leu Ser Met Pro  Gln Val Asn Ile Val  Lys Lys Thr
    1085                1090                1095

gag gtg  cag aca ggc ggc ttc  agc aaa gag tct atc  ctg ccc aag      3339
Glu Val  Gln Thr Gly Gly Phe  Ser Lys Glu Ser Ile  Leu Pro Lys
    1100                1105                1110

agg aac  agc gat aag ctg atc  gcc aga aag aag gac  tgg gac cct      3384
Arg Asn  Ser Asp Lys Leu Ile  Ala Arg Lys Lys Asp  Trp Asp Pro
    1115                1120                1125

aag aag  tac ggc ggc ttc gac  agc ccc acc gtg gcc  tat tct gtg      3429
Lys Lys  Tyr Gly Gly Phe Asp  Ser Pro Thr Val Ala  Tyr Ser Val
    1130                1135                1140

ctg gtg  gtg gcc aaa gtg gaa  aag ggc aag tcc aag  aaa ctg aag      3474
Leu Val  Val Ala Lys Val Glu  Lys Gly Lys Ser Lys  Lys Leu Lys
    1145                1150                1155

agt gtg  aaa gag ctg ctg ggg  atc acc atc atg gaa  aga agc agc      3519
Ser Val  Lys Glu Leu Leu Gly  Ile Thr Ile Met Glu  Arg Ser Ser
    1160                1165                1170

ttc gag  aag aat ccc atc gac  ttt ctg gaa gcc aag  ggc tac aaa      3564
Phe Glu  Lys Asn Pro Ile Asp  Phe Leu Glu Ala Lys  Gly Tyr Lys
    1175                1180                1185

gaa gtg  aaa aag gac ctg atc  atc aag ctg cct aag  tac tcc ctg      3609
Glu Val  Lys Lys Asp Leu Ile  Ile Lys Leu Pro Lys  Tyr Ser Leu
    1190                1195                1200

ttc gag  ctg gaa aac ggc cgg  aag aga atg ctg gcc  tct gcc ggc      3654
Phe Glu  Leu Glu Asn Gly Arg  Lys Arg Met Leu Ala  Ser Ala Gly
    1205                1210                1215

gaa ctg  cag aag gga aac gaa  ctg gcc ctg ccc tcc  aaa tat gtg      3699
Glu Leu  Gln Lys Gly Asn Glu  Leu Ala Leu Pro Ser  Lys Tyr Val
    1220                1225                1230

aac ttc  ctg tac ctg gcc agc  cac tat gag aag ctg  aag ggc tcc      3744
Asn Phe  Leu Tyr Leu Ala Ser  His Tyr Glu Lys Leu  Lys Gly Ser
    1235                1240                1245

ccc gag  gat aat gag cag aaa  cag ctg ttt gtg gaa  cag cac aag      3789
Pro Glu  Asp Asn Glu Gln Lys  Gln Leu Phe Val Glu  Gln His Lys
    1250                1255                1260

cac tac  ctg gac gag atc atc  gag cag atc agc gag  ttc tcc aag      3834
His Tyr  Leu Asp Glu Ile Ile  Glu Gln Ile Ser Glu  Phe Ser Lys
    1265                1270                1275

aga gtg  atc ctg gcc gac gct  aat ctg gac aaa gtg  ctg tcc gcc      3879
Arg Val  Ile Leu Ala Asp Ala  Asn Leu Asp Lys Val  Leu Ser Ala
    1280                1285                1290

tac aac  aag cac cgg gat aag  ccc atc aga gag cag  gcc gag aat      3924
Tyr Asn  Lys His Arg Asp Lys  Pro Ile Arg Glu Gln  Ala Glu Asn
    1295                1300                1305

atc atc  cac ctg ttt acc ctg  acc aat ctg gga gcc  cct gcc gcc      3969
Ile Ile  His Leu Phe Thr Leu  Thr Asn Leu Gly Ala  Pro Ala Ala
    1310                1315                1320

ttc aag  tac ttt gac acc acc  atc gac cgg aag agg  tac acc agc      4014
Phe Lys  Tyr Phe Asp Thr Thr  Ile Asp Arg Lys Arg  Tyr Thr Ser
    1325                1330                1335

acc aaa  gag gtg ctg gac gcc  acc ctg atc cac cag  agc atc acc      4059
Thr Lys  Glu Val Leu Asp Ala  Thr Leu Ile His Gln  Ser Ile Thr
    1340                1345                1350

ggc ctg  tac gag aca cgg atc  gac ctg tct cag ctg  gga ggc gac      4104
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Gly Leu  Tyr Glu Thr Arg Ile  Asp Leu Ser Gln Leu  Gly Gly Asp
    1355                1360                1365


<210> SEQ ID NO 502
<211> LENGTH: 1368
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 502

Met Asp Lys Lys Tyr Ser Ile Gly Leu Asp Ile Gly Thr Asn Ser Val
1               5                   10                  15

Gly Trp Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe
            20                  25                  30

Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile
        35                  40                  45

Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
    50                  55                  60

Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
65                  70                  75                  80

Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser
                85                  90                  95

Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
            100                 105                 110

His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr
        115                 120                 125

His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp
    130                 135                 140

Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
145                 150                 155                 160

Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
                165                 170                 175

Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr
            180                 185                 190

Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp Ala
        195                 200                 205

Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn
    210                 215                 220

Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn
225                 230                 235                 240

Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe
                245                 250                 255

Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp
            260                 265                 270

Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp
        275                 280                 285

Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp
    290                 295                 300

Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser
305                 310                 315                 320

Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys
                325                 330                 335

Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe
            340                 345                 350
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Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser
        355                 360                 365

Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp
    370                 375                 380

Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg
385                 390                 395                 400

Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu
                405                 410                 415

Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe
            420                 425                 430

Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile
        435                 440                 445

Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp
    450                 455                 460

Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu
465                 470                 475                 480

Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr
                485                 490                 495

Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser
            500                 505                 510

Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys
        515                 520                 525

Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln
    530                 535                 540

Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr
545                 550                 555                 560

Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp
                565                 570                 575

Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly
            580                 585                 590

Thr Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp
        595                 600                 605

Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr
    610                 615                 620

Leu Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala
625                 630                 635                 640

His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr
                645                 650                 655

Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp
            660                 665                 670

Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe
        675                 680                 685

Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr Phe
    690                 695                 700

Lys Glu Asp Ile Gln Lys Ala Gln Val Ser Gly Gln Gly Asp Ser Leu
705                 710                 715                 720

His Glu His Ile Ala Asn Leu Ala Gly Ser Pro Ala Ile Lys Lys Gly
                725                 730                 735

Ile Leu Gln Thr Val Lys Val Val Asp Glu Leu Val Lys Val Met Gly
            740                 745                 750

Arg His Lys Pro Glu Asn Ile Val Ile Ala Met Ala Arg Glu Asn Gln
        755                 760                 765
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Thr Thr Gln Lys Gly Gln Lys Asn Ser Arg Glu Arg Met Lys Arg Ile
    770                 775                 780

Glu Glu Gly Ile Lys Glu Leu Gly Ser Gln Ile Leu Lys Glu His Pro
785                 790                 795                 800

Val Glu Asn Thr Gln Leu Gln Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu
                805                 810                 815

Gln Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg
            820                 825                 830

Leu Ser Asp Tyr Asp Val Asp Ala Ile Val Pro Gln Ser Phe Leu Lys
        835                 840                 845

Asp Asp Ser Ile Asp Ala Lys Val Leu Thr Arg Ser Asp Lys Ala Arg
    850                 855                 860

Gly Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys
865                 870                 875                 880

Asn Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys
                885                 890                 895

Phe Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp
            900                 905                 910

Lys Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr
        915                 920                 925

Lys His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp
    930                 935                 940

Glu Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser
945                 950                 955                 960

Lys Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg
                965                 970                 975

Glu Ile Asn Asn Tyr His His Ala His Ala Ala Tyr Leu Asn Ala Val
            980                 985                 990

Val Gly Thr Ala Leu Ile Lys Lys  Tyr Pro Lys Leu Glu  Ser Glu Phe
        995                 1000                1005

Val Tyr  Gly Asp Tyr Lys Val  Tyr Asp Val Arg Lys  Met Ile Ala
    1010                1015                1020

Lys Ser  Glu Gln Glu Ile Gly  Lys Ala Thr Ala Lys  Tyr Phe Phe
    1025                1030                1035

Tyr Ser  Asn Ile Met Asn Phe  Phe Lys Thr Glu Ile  Thr Leu Ala
    1040                1045                1050

Asn Gly  Glu Ile Arg Lys Arg  Pro Leu Ile Glu Thr  Asn Gly Glu
    1055                1060                1065

Thr Gly  Glu Ile Val Trp Asp  Lys Gly Arg Asp Phe  Ala Thr Val
    1070                1075                1080

Arg Lys  Val Leu Ser Met Pro  Gln Val Asn Ile Val  Lys Lys Thr
    1085                1090                1095

Glu Val  Gln Thr Gly Gly Phe  Ser Lys Glu Ser Ile  Leu Pro Lys
    1100                1105                1110

Arg Asn  Ser Asp Lys Leu Ile  Ala Arg Lys Lys Asp  Trp Asp Pro
    1115                1120                1125

Lys Lys  Tyr Gly Gly Phe Asp  Ser Pro Thr Val Ala  Tyr Ser Val
    1130                1135                1140

Leu Val  Val Ala Lys Val Glu  Lys Gly Lys Ser Lys  Lys Leu Lys
    1145                1150                1155

Ser Val  Lys Glu Leu Leu Gly  Ile Thr Ile Met Glu  Arg Ser Ser
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    1160                1165                1170

Phe Glu  Lys Asn Pro Ile Asp  Phe Leu Glu Ala Lys  Gly Tyr Lys
    1175                1180                1185

Glu Val  Lys Lys Asp Leu Ile  Ile Lys Leu Pro Lys  Tyr Ser Leu
    1190                1195                1200

Phe Glu  Leu Glu Asn Gly Arg  Lys Arg Met Leu Ala  Ser Ala Gly
    1205                1210                1215

Glu Leu  Gln Lys Gly Asn Glu  Leu Ala Leu Pro Ser  Lys Tyr Val
    1220                1225                1230

Asn Phe  Leu Tyr Leu Ala Ser  His Tyr Glu Lys Leu  Lys Gly Ser
    1235                1240                1245

Pro Glu  Asp Asn Glu Gln Lys  Gln Leu Phe Val Glu  Gln His Lys
    1250                1255                1260

His Tyr  Leu Asp Glu Ile Ile  Glu Gln Ile Ser Glu  Phe Ser Lys
    1265                1270                1275

Arg Val  Ile Leu Ala Asp Ala  Asn Leu Asp Lys Val  Leu Ser Ala
    1280                1285                1290

Tyr Asn  Lys His Arg Asp Lys  Pro Ile Arg Glu Gln  Ala Glu Asn
    1295                1300                1305

Ile Ile  His Leu Phe Thr Leu  Thr Asn Leu Gly Ala  Pro Ala Ala
    1310                1315                1320

Phe Lys  Tyr Phe Asp Thr Thr  Ile Asp Arg Lys Arg  Tyr Thr Ser
    1325                1330                1335

Thr Lys  Glu Val Leu Asp Ala  Thr Leu Ile His Gln  Ser Ile Thr
    1340                1345                1350

Gly Leu  Tyr Glu Thr Arg Ile  Asp Leu Ser Gln Leu  Gly Gly Asp
    1355                1360                1365


<210> SEQ ID NO 503
<211> LENGTH: 15
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 503

cagaagaaga agggc                                                    15


<210> SEQ ID NO 504
<211> LENGTH: 51
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 504

ccaatgggga ggacatcgat gtcacctcca atgactaggg tggtgggcaa c             51


<210> SEQ ID NO 505
<211> LENGTH: 15
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 505

ctctggccac tccct                                                    15


<210> SEQ ID NO 506
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
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<400> SEQUENCE: 506

acatcgatgt cacctccaat gacaagcttg ctagcggtgg gcaaccacaa ac 52

<210> SEQ ID NO 507
<211> LENGTH: 1733
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 507

ccgtttaaac aattctgcag gaatctagtt attaatagta atcaattacg gggtcattag 60

ttcatagccc atatatggag ttccgcgtta cataacttac ggtaaatggc ccgcctggct 120

gaccgcccaa cgacccccgc ccattgacgt caataatgac gtatgttccc atagtaacgc 180

caatagggac tttccattga cgtcaatggg tggagtattt acggtaaact gcccacttgg 240

cagtacatca agtgtatcat atgccaagta cgcccccta tgacgtcaat gacggtaaat 300

ggcccgcctg gcattatgcc cagtacatga ccttatggga ctttcctact tggcagtaca 360

tctacgtatt agtcatcgct attaccatgg tcgaggtgag ccccacgttc tgcttcactc 420

tccccatctc ccccccctcc ccacccccaa ttttgtattt atttatttttt taattatttt 480

gtgcagcgat gggggcgggg ggggggggggg ggcgcgcgcc aggcggggcg gggcggggcg 540

aggggcgggg cggggcgagg cggagaggtg cggcggcagc caatcagagc ggcgcgctcc 600

gaaagtttcc ttttatggcg aggcggcggc ggcggcggcc ctataaaaag cgaagcgcgc 660

ggcgggcgga agtcgctgcg cgctgccttc gccccgtgcc ccgctccgcc gccgcctcgc 720

gccgcccgcc ccggctctga ctgaccgcgt tactcccaca ggtgagcggg cgggacggcc 780

cttctcctcc gggctgtaat tagcgcttgg tttaatgacg gcttgtttct tttctgtggc 840

tgcgtgaaag ccttgagggg ctccgggagg gccctttgtg cgggggggagc ggctcggggg 900

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<210> SEQ ID NO 508
<211> LENGTH: 4269
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 508

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gacaagaagt acagcatcgg cctggacatc ggcaccaact ctgtgggctg ggccgtgatc 180

accgacgagt acaaggtgcc cagcaagaaa ttcaaggtgc tgggcaacac cgaccggcac 240

agcatcaaga agaacctgat cggagccctg ctgttcgaca gcggcgaaac agccgaggcc 300

acccggctga agagaaccgc cagaagaaga tacaccagac ggaagaaccg gatctgctat 360

ctgcaagaga tcttcagcaa cgagatggcc aaggtggacg acagcttctt ccacagactg 420

gaagagtcct tcctggtgga agaggataag aagcacgagc ggcaccccat cttcggcaac 480

atcgtggacg aggtggccta ccacgagaag taccccacca tctaccacct gagaaagaaa 540

ctggtggaca gcaccgacaa ggccgacctg cggctgatct atctggccct ggcccacatg 600

atcaagttcc ggggccactt cctgatcgag ggcgacctga accccgacaa cagcgacgtg 660

gacaagctgt tcatccagct ggtgcagacc tacaaccagc tgttcgagga aaaccccatc 720

aacgccagcg gcgtggacgc caaggccatc ctgtctgcca gactgagcaa gagcagacgg 780

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tatgagaagc tgaagggctc ccccgaggat aatgagcaga aacagctgtt tgtggaacag 3900

cacaagcact acctggacga gatcatcgag cagatcagcg agttctccaa gagagtgatc 3960

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gtgctggacg ccaccctgat ccaccagagc atcaccggcc tgtacgagac acggatcgac 4200

ctgtctcagc tgggaggcga caaaaggccg gcggccacga aaaaggccgg ccaggcaaaa 4260

aagaaaaag 4269

<210> SEQ ID NO 509
<211> LENGTH: 780

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 509

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gacggcgacg taaacggcca caagttcagc gtgtccggcg agggcgaggg cgatgccacc 180

tacggcaagc tgaccctgaa gttcatctgc accaccggca agctgcccgt gccctggccc 240

accctcgtga ccaccctgac ctacggcgtg cagtgcttca gccgctaccc cgaccacatg 300

aagcagcacg acttcttcaa gtccgccatg cccgaaggct acgtccagga gcgcaccatc 360

ttcttcaagg acgacggcaa ctacaagacc cgcgccgagg tgaagttcga gggcgacacc 420

ctggtgaacc gcatcgagct gaagggcatc gacttcaagg aggacggcaa catcctgggg 480

cacaagctgg agtacaacta caacagccac aacgtctata tcatggccga caagcagaag 540

aacggcatca aggtgaactt caagatccgc cacaacatcg aggacggcag cgtgcagctc 600

gccgaccact accagcagaa cacccccatc ggcgacggcc ccgtgctgct gcccgacaac 660

cactacctga gcacccagtc cgccctgagc aaagacccca acgagaagcg cgatcacatg 720

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<210> SEQ ID NO 510
<211> LENGTH: 597
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 510

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ccgtatggct ttcattttct cctccttgta taaatcctgg ttgctgtctc tttatgagga 180

gttgtggccc gttgtcaggc aacgtggcgt ggtgtgcact gtgtttgctg acgcaacccc 240

cactggttgg ggcattgcca ccacctgtca gctcctttcc gggactttcg ctttcccct 300

ccctattgcc acggcggaac tcatcgccgc ctgccttgcc cgctgctgga caggggctcg 360

gctgttgggc actgacaatt ccgtggtgtt gtcggggaaa tcatcgtcct ttccttggct 420

gctcgcctgt gttgccacct ggattctgcg cgggacgtcc ttctgctacg tcccttcggc 480

cctcaatcca gcggaccttc cttcccgcgg cctgctgccg gctctgcggc ctcttccgcg 540

tcttcgcctt cgccctcaga cgagtcggat ctccctttgg gccgcctccc cgcatcg 597

<210> SEQ ID NO 511
<211> LENGTH: 210
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 511

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cgcattgtct gagtaggtgt cattctattc tgggggggtgg ggtggggcag gacagcaagg 180

gggaggattg ggaagacaat ggcaggcatg 210

<210> SEQ ID NO 512
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (109)..(109)
<223> OTHER INFORMATION: a, c, t, g, unknown or other
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (135)..(135)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 512

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gatctgcgac tctagaggat ctgcgactct agaggatcat aatcagccnt accacatttt 120

gtagaggttt tactngcttt aaaaaacctc ccacacctcc ccctgaacct gaaacataaa 180

atgaatgcaa ttgttgttgt taacttgttt attgcagctt ataatggtta caaataaagc 240

aatagcatca caaatttcac aaataaagca ttttttttcac tgcattctag ttgtggtttg 300

tccaaactca tcaatgtatc ttatcatgtc tggatctgcg actctagagg atcataatca 360

gccataccac atttgtagag gttttacttg ctttaaaaaa cctcccacac ctccccctga 420

acctgaaaca taaaatgaat gcaattgttg ttgttaactt gtttattgca gcttataatg 480

gttacaaata aagcaatagc atcacaaatt tcacaaataa agcatttttt tcactgcatt 540

ctagttgtgg tttgtccaaa ctcatcaatg tatcttatca tgtctggatc tgcgactcta 600

gaggatcata atcagccata ccacatttgt agaggtttta cttgctttaa aaaacctccc 660

acacctcccc ctgaacctga aacataaaat gaatgcaatt gttgttgtta acttgtttat 720

tgcagcttat aatggttaca aataaagcaa tagcatcaca aatttcacaa ataaagcatt 780

tttttcactg cattctagtt gtggtttgtc caaactcatc aatgtatctt atcatgtctg 840

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agttat 906

<210> SEQ ID NO 513
<211> LENGTH: 1079
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 513

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gggagaaggg agcggaaaag tctccaccgg acgcggccat ggctcggggg ggggggggca 180

gcggaggagc gcttccggcc gacgtctcgt cgctgattgg cttcttttcc tcccgccgtg 240

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tgtgaaaaca caaatggcgt gttttggttg gcgtaaggcg cctgtcagtt aacggcagcc 300

ggagtgcgca gccgccggca gcctcgctct gcccactggg tggggcggga ggtaggtggg 360

gtgaggcgag ctggacgtgc gggcgcggtc ggcctctggc ggggcggggg aggggaggga 420

gggtcagcga aagtagctcg cgcgcgagcg gccgcccacc ctccccttcc tctgggggag 480

tcgttttacc cgccgccggc cgggcctcgt cgtctgattg gctctcgggg cccagaaaac 540

tggcccttgc cattggctcg tgttcgtgca agttgagtcc atccgccggc cagcgggggc 600

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<210> SEQ ID NO 514
<211> LENGTH: 4336
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 514

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tgtcgggaag ttttttaata ggggcaaata aggaaaatgg gaggataggt agtcatctgg 180

ggttttatgc agcaaaacta caggttatta ttgcttgtga tccgcctcgg agtattttcc 240

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ctacagtatg aaattacagt gtcgcgagtt agactatgta agcagaattt taatcatttt 360

taaagagccc agtacttcat atccatttct cccgctcctt ctgcagcctt atcaaaaggt 420

attttagaac actcatttta gccccatttt catttattat actggcttat ccaaccccta 480

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acagattagt tacatacacc acaaatcgag gctgtagctg gggcctcaac actgcagttc 600

ttttataact ccttagtaca ctttttgttg atcctttgcc ttgatcctta attttcagtg 660

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tttacaacaa tagatgtatt gagaatccaa cctaaagctt aactttccac tcccatgaat 780

gcctctctcc ttttttctcca tttataaact gagctattaa ccattaatgg tttccaggtg 840

gatgtctcct cccccaatat tacctgatgt atcttacata ttgccaggct gatattttaa 900

gacattaaaa ggtatatttc attattgagc cacatggtat tgattactgc ttactaaaat 960

tttgtcattg tacacatctg taaaaggtgg ttccttttgg aatgcaaagt tcaggtgttt 1020

gttgtctttc ctgacctaag gtcttgtgag cttgtatttt ttctatttaa gcagtgcttt 1080

ctcttggact ggcttgactc atggcattct acacgttatt gctggtctaa atgtgatttt 1140

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ttaagcaaca aatgtatttg tgaagcttgg tttttaggtt gttgtgttgt gtgtgcttgt 1260

gctctataat aatactatcc aggggctgga gaggtggctc ggagttcaag agcacagact 1320

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gcagccatta ctaaacatga aagcaagtaa tgataatttt ggatttcaaa aatgtagggc 1860

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cctaccttag gctgattgtt gttcaaataa acttaagttt cctgtcaggt gatgtcatat 3360

gatttcatat atcaaggcaa aacatgttat atatgttaaa catttgtact taatgtgaaa 3420

gttaggtctt tgtgggtttg atttttaatt ttcaaaacct gagctaaata agtcattttt 3480

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acatgtctta catttggtgg aattgtataa ttgtggtttg caggcaagac tctctgacct 3540

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ccaaatgtgt cagtttcata gcctgaagaa cgagatcagc agcctctgtt ccacatacac 1800

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What is claimed is:

1. A non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences (NLSs) in the proximity of a terminus of the CRISPR enzyme,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence,

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, and

wherein the chimeric RNA polynucleotide sequence comprises two or more hairpins.

2. A multiplexed CRISPR enzyme system, wherein the system comprises a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell,

(h) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme

comprising at least one or more nuclear localization sequences (NLSs) in the proximity of a terminus of the CRISPR enzyme,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence,

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

wherein the chiRNA polynucleotide sequence comprises two or more hairpins, and

wherein in the multiplexed system multiple chiRNA polynucleotide sequences are used.

3. The composition of claim **1** or **2**, wherein the first regulatory element is a polymerase III promoter.

4. The composition of claim **1** or **2**, wherein the second regulatory element is a polymerase II promoter.

5. The composition of claim **1** or **2**, wherein the CRISPR enzyme comprises one or more NLSs of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell.

6. The composition of claim **1** or **2**, wherein the tracr sequence exhibits at least 50% of sequence complementarity along the length of the tracr mate sequence when optimally aligned.

7. The composition of claim **1** or **2**, wherein the CRISPR enzyme is a type II CRISPR system enzyme.

8. The composition of claim **1** or **2**, wherein the CRISPR enzyme is a Cas9 enzyme.

9. The composition of claim **1** or **2**, wherein the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell.

10. The composition of claim **1** or **2**, wherein the guide sequence is at least 15 nucleotides in length.

11. The composition of claim **1** or **2**, wherein the chimeric RNA polynucleotide sequence comprises two, three, four or five hairpins

12. A non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, and

(b) a tracr mate sequence,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences (NLSs) in the proximity of a terminus of the CRISPR enzyme, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence.

13. A multiplexed CRISPR enzyme system, wherein the system comprises a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, and

(b) tracr mate sequence,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences (NLSs) in the proximity of a terminus of the CRISPR enzyme, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence,

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, and

wherein in the multiplexed system multiple guide sequences and a single tracr sequence is used.

14. The composition of claim **12** or **13**, wherein the first regulatory element is a polymerase III promoter.

15. The composition of claim **12** or **13**, wherein the second regulatory element is a polymerase II promoter.

16. The composition of claim **12** or **13**, wherein the third regulatory element is a polymerase III promoter.

17. The composition of claim **12** or **13**, wherein the CRISPR enzyme comprises one or more NLSs of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell.

18. The composition of claim **12** or **13**, wherein the tracr sequence exhibits at least 50% of sequence complementarity along the length of the tracr mate sequence when optimally aligned.

19. The composition of claim **12** or **13**, wherein the CRISPR enzyme is a type II CRISPR system enzyme.

20. The composition of claim **12** or **13**, wherein the CRISPR enzyme is a Cas9 enzyme.

21. The composition of claim **12** or **13**, wherein the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell.

22. The composition of claim **12** or **13**, wherein the guide sequence is at least 15 nucleotides in length.

23. A eukaryotic host cell comprising the composition of any of the preceding claims.

24. An organism comprising the eukaryotic host cell of claim **23**.

25. A non-human organism comprising the eukaryotic host cell of claim **23**.

26. A kit comprising the composition of any of claims **1** to **22** and instructions for using said kit.

27. A method of altering the expression of a genomic locus of interest in a eukaryotic cell comprising

contacting the genomic locus with the composition of any of claims **1** to **22**, and

determining if the expression of the genomic locus has been altered.

28. The method of claim **27** wherein the guide sequence directs sequence-specific binding of the CRISPR complex to the target sequence based on the presence of a CRISPR motif sequence.

29. The method of claim **28**, wherein the CRISPR motif sequence is NAG.

30. The method of selecting one or more prokaryotic cell(s) by introducing one or more mutations in a gene in the one or more prokaryotic cell (s), the method comprising:

introducing one or more vectors into the prokaryotic cell (s), wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, a tracr sequence, and an editing template;

wherein the editing template comprises the one or more mutations that abolish CRISPR enzyme cleavage;

allowing homologous recombination of the editing template with the target polynucleotide in the cell(s) to be selected;

allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

wherein binding of the CRISPR complex to the target polynucleotide induces cell death,

thereby allowing one or more prokaryotic cell(s) in which one or more mutations have been introduced to be selected.

31. The method of claim **30**, wherein the CRISPR enzyme is a type II CRISPR system enzyme

32. The method of claim **31**, wherein the CRISPR enzyme is Cas9.

33. A vector system comprising one or more vectors, wherein the system comprises

a. a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting a guide sequence upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and

b. a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence;

wherein components (a) and (b) are located on the same or different vectors of the system.

34. The vector system of claim **33**, wherein component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element.

35. The vector system of claim **33**, wherein component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell.

36. The vector system of claim **35**, wherein the system comprises the tracr sequence under the control of a third regulatory element.

37. The vector system of claim **33**, wherein the tracr sequence exhibits at least 50% of sequence complementarity along the length of the tracr mate sequence when optimally aligned.

38. The vector system of claim **33**, wherein the CRISPR enzyme comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell.

39. The vector system of claim **33**, wherein the CRISPR enzyme is a type II CRISPR system enzyme.

40. The vector system of claim **33**, wherein the CRISPR enzyme is a Cas9 enzyme.

41. The vector system of claim **33**, wherein the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell.

42. The vector system of claim **33**, wherein the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence.

43. The vector system of claim **33**, wherein the CRISPR enzyme lacks DNA strand cleavage activity.

44. The vector system of claim **33**, wherein the first regulatory element is a polymerase III promoter.

45. The vector system of claim **33**, wherein the second regulatory element is a polymerase II promoter.

46. The vector system of claim **36**, wherein the third regulatory element is a polymerase III promoter.

47. The vector system of claim **33**, wherein the guide sequence is at least 15 nucleotides in length.

48. The vector system of claim **33**, wherein fewer than 50% of the nucleotides of the guide sequence participate in self-complementary base-pairing when optimally folded.

49. A vector comprising a regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising one or more nuclear localization sequences, wherein said regulatory element drives transcription of the CRISPR enzyme in a eukaryotic cell such that said CRISPR enzyme accumulates in a detectable amount in the nucleus of the eukaryotic cell.

50. The vector of claim **49**, wherein said regulatory element is a polymerase II promoter.

51. The vector of claim **49**, wherein said CRISPR enzyme is a type II CRISPR system enzyme.

52. The vector of claim **49**, wherein said CRISPR enzyme is a Cas9 enzyme.

53. A method of modifying a target polynucleotide in a eukaryotic cell, the method comprising allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

54. The method of claim **53**, wherein said cleavage comprises cleaving two strands at the location of the target sequence by said CRISPR enzyme.

55. The method of claim **53**, wherein said cleavage results in decreased transcription of a target gene.

56. The method of claim **53**, further comprising repairing said cleaved target polynucleotide by homologous recombi-

nation with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide.

57. The method of claim **56**, wherein said mutation results in one or more amino acid changes in a protein expressed from a gene comprising the target sequence.

58. The method of claim **53**, further comprising delivering one or more vectors to said eukaryotic cell, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence.

59. The method of claim **58**, wherein said vectors are delivered to the eukaryotic cell in a subject.

60. The method of claim **53**, wherein said modifying takes place in said eukaryotic cell in a cell culture.

61. The method of claim **53**, further comprising isolating said eukaryotic cell from a subject prior to said modifying.

62. The method of claim **61**, further comprising returning said eukaryotic cell and/or cells derived therefrom to said subject.

* * * * *