(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 April 2017 (06.04.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/059313 A1

(51) International Patent Classification:
C12N 15/10 (2006.01) *C12Q 1/68* (2006.01)

(21) International Application Number:
PCT/US2016/054912

(22) International Filing Date:
30 September 2016 (30.09.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/235,154 30 September 2015 (30.09.2015) US

(71) Applicant: **THE GENERAL HOSPITAL CORPORATION** [US/US]; 55 Fruit Street, Boston, Massachusetts 02114 (US).

(72) Inventors: **JOUNG, J. Keith**; 1 Magnolia Way, Winchester, Massachusetts 01890 (US). **TSAI, Shengdar**; 1353 Vinton Ave., Memphis, TN 38104 (US).

(74) Agents: **DEYOUNG, Janice Kugler** et al.; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,

[Continued on next page]

(54) Title: COMPREHENSIVE IN VITRO REPORTING OF CLEAVAGE EVENTS BY SEQUENCING (CIRCLE-SEQ)

(57) Abstract: Sensitive, unbiased methods for genome-wide detection of potential CRISPR-Cas9 off-target cleavage sites from cell type-specific genomic DNA samples.

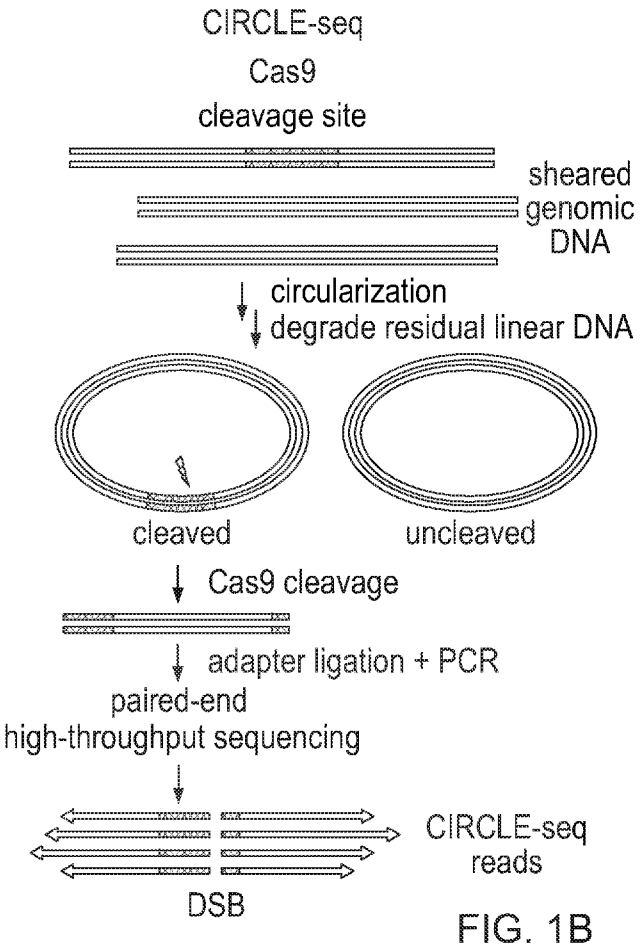


FIG. 1B

WO 2017/059313 A1

SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

Comprehensive In vitro Reporting of Cleavage Events by Sequencing (CIRCLE-seq)

CLAIM OF PRIORITY

This application claims the benefit of U.S. Patent Application Serial No. 62/235,154, filed on September 30, 2015. The entire contents of the foregoing are hereby incorporated by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant No. DP1 GM105378 awarded by the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

Described herein are *in vitro* methods for defining the genome-wide cleavage specificities of engineered nucleases such as CRISPR-Cas9 Nucleases.

BACKGROUND

Engineered nuclease technology including zinc fingers, TALENs, and CRISPR-Cas9 nucleases, is revolutionizing biomedical research and providing important new modalities for therapy of gene-based diseases. Sensitive detection of off-target effects is important for translating these methods into human therapeutics. In vitro biochemical methods for finding off-targets offer potential advantages of greater reproducibility and scalability while avoiding limitations associated with strategies that require the culture and manipulation of living cells.

SUMMARY

At least in part, the present invention is based on the development of sensitive, unbiased methods for genome-wide detection of potential engineered nuclease (e.g., CRISPR-Cas9) off-target cleavage sites from cell type-specific genomic DNA samples. The present methods use exonuclease selection of covalently closed DNA molecules to create a population of genomic DNA molecules with very few free DNA ends, as a starting population for cleavage-specific enrichment and sequencing. Enrichment of these cleaved fragments, estimated to be >500,000X from human genomic DNA, enables very sequencing-efficient discovery of in vitro cleaved DNA

fragments, in contrast to methods such as Digenome-Seq (Kim et al., Nat Methods. 2015 Mar;12(3):237-43) that rely on whole-genome sequencing and that have much higher background. After optimization, the present *in vitro* assay detected 100% of off-target cleavage sites detected by the in-cell GUIDE-seq assay at *the VEGFA site 1* and *EMX1* target sites performed in human U2OS cells and described in Tsai et al., Nat Biotechnol. 2015 Feb;33(2):187-97 as well as additional new off-target sites not found by the GUIDE-seq experiments (in other words, the present in vitro assay detects a superset of GUIDE-seq detected cleavage sites).

Described herein are methods of enzymatically preparing a library of covalently closed fully circularized DNA fragments with minimized numbers of DNA double-stranded breaks (DSBs) by ligation of stem-loop or hairpin adapters, exonuclease selection, enzymatic opening of the stem-loop and repair of ends, and intramolecular ligation. These circles can then be manipulated to detect nuclease-induced cleavage of this enzymatically purified library of covalently closed fully circularized DNA fragments by sequencing. Together, these two methods comprise a strategy for efficiently mining for nuclease-induced cleavage sites in complex mixtures of DNA. Thus, the methods can include creating a population of DNA molecules without ends, treating that population with a nuclease, and finding DNA molecules in this population that have newly created ends as a result of nuclease-induced cleavage.

Thus, provided herein are methods for preparing a library of covalently closed circular double-stranded DNA fragments. The methods can include providing dsDNA, e.g., genomic DNA (gDNA) from a cell type or organism of interest or synthetic DNA; randomly shearing the DNA to a defined average length, e.g., an average length of about 200-500 bps, e.g., about 300 bps, to provide a population of DNA fragments; optionally preparing the fragments for end-ligation, e.g., by end-repairing and/or A-tailing the sheared DNA; ligating to the ends of the fragments a stem-loop adapter comprising at least a single deoxyuridine adjacent to or within a loop sequence comprising a palindromic sequence, to prepare a population of ligated linear dsDNA fragments; contacting the library with an exonuclease (e.g., a cocktail such as Lambda exonuclease and/or E. coli Exonuclease I) to degrade any remaining linear fragments with unligated ends, to produce a purified population of ligated linear dsDNA fragments; contacting the library with enzymes that nick the ligated dsDNA fragments at the deoxyuridine and to remove a 3' terminal phosphate, e.g., with uracil

DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase, to nick the DNA at the deoxyuridine and T4 Polynucleotide Kinase to remove a 3' terminal phosphate; incubating the nicked linear dsDNA fragments under conditions sufficient to promote intramolecular ligation and formation of circular DNA molecules; and purifying the ligated fragments using an exonuclease, thereby preparing a library of covalently closed fully circular double-stranded DNA fragments.

In some embodiments, the methods include contacting the library of covalently closed fully circular dsDNA fragments with a nuclease (e.g., an engineered nuclease as described herein) to induce site-specific cleavage; optionally preparing the cleaved fragments for end-ligation, e.g., by end-repairing and/or A-tailing the sheared DNA ligating a sequencing adapter comprising at least (and preferably only) a single deoxyuridine and a primer site compatible for use in PCR priming and/or sequencing at the cleavage site; contacting the library with an enzyme, e.g., uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase, that nicks at the deoxyuridine; and sequencing those fragments using the sequencing adapter.

Also provided herein are methods for preparing a library of fragments comprising nuclease-induced double stranded breaks in dsDNA, e.g., genomic DNA (gDNA). The methods can include providing dsDNA, e.g., gDNA from a cell type or organism of interest; randomly shearing the dsDNA to a defined average length, e.g., an average length of about 200-500 bps, e.g., about 300 bps; optionally end-repairing and/or A-tailing the sheared DNA; ligating a first stem-loop adapter, preferably comprising a first region, e.g., of about 10-15, e.g., about 12 nucleotides; a second region preferably of about 5 nucleotides, that forms one or more loops and comprises a single deoxyuridine nucleotide adjacent to a palindromic sequence for intramolecular ligation; and a third region that is complementary to the first region with one additional nucleotide, e.g., about 13 nucleotides; contacting the library with uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase, to nick the dsDNA at the deoxyuridine and T4 Polynucleotide Kinase to remove a terminal 3' phosphate that is molecularly incompatible with ligation; incubating the nicked dsDNA under conditions sufficient to promote intramolecular ligation and formation of a sample comprising circular dsDNA molecules; contacting the sample with one or more exonucleases (e.g., bacteriophage lambda exonuclease, E. coli ExoI, PlasmidSafe™ ATP-dependent exonuclease), sufficient to degrade any dsDNA molecules that are not circular; treating the sample with a nuclease to induce site-

specific cleavage (e.g., of on- and/or off-target sites, e.g., to induce blunt or staggered/overhanging ends) optionally end-repairing and then A-tailing the resulting ends; ligating a sequencing adapter comprising a first region of about 12 nucleotides; a second region of about 40 nucleotides that forms a loop, e.g., one or more hairpin loops, and comprises a second primer compatible for use in PCR priming and/or sequencing, e.g., next generation sequencing (NGS); and a third region of about 13 nucleotides (e.g., one longer than the first region) that is complementary to the first region; and a single deoxyuridine nucleotide between the second and third regions, to create a population wherein the DNA fragments that were cleaved by the nuclease have a sequencing adapter ligated to the ends; thereby preparing a library of fragments enriched for nuclease-cleaved adapter-ligated fragments, e.g., wherein each end was created by a nuclease-induced double stranded break in the dsDNA and then ligated with an adapter.

In some embodiments, the methods include contacting the library with uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase to nick the DNA at the deoxyuridine; and sequencing those fragments bearing a sequencing adapter.

In some embodiments, the exonuclease used to degrade any remaining linear fragments with unligated ends is a cocktail of nucleases comprising one or more of bacteriophage Lambda exonuclease, *E. coli* Exonuclease I, and an ATP-dependent exonuclease.

In some embodiments, the engineered nuclease is selected from the group consisting of meganucleases, MegaTALs, zinc-finger nucleases, transcription activator effector-like nucleases (TALEN), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas RNA-guided nucleases (CRISPR/Cas RGNs), and FokI-dCas9 fusion proteins.

In some embodiments of the methods described herein, treating the sample with a nuclease to induce site-specific cleavage, e.g., at on- and off-target sites, comprises contacting the sample with a Cas9 nuclease complexed with a specific guide RNA (gRNA).

In some embodiments, the primer site in the hairpin comprises a next generation sequencing primer site, a randomized DNA barcode or unique molecular identifier (UMI).

In some embodiments, the methods include contacting the library with uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase to nick the DNA at the deoxyuridine; and sequencing those fragments bearing a first and a second hairpin adapter.

In some embodiments, the engineered nuclease is selected from the group consisting of meganucleases, MegaTALs, zinc-finger nucleases, transcription activator effector-like nucleases (TALEN), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas RNA-guided nucleases (CRISPR/Cas RGNs), and FokI-dCas9 fusions (RNA-guided FokI nuclease).

In some embodiments, treating the sample with a nuclease to induce site-specific cleavage, e.g., at on- and off-target sites, comprises contacting the sample with a Cas9 nuclease complexed with a specific guide RNA (gRNA).

In some embodiments, the DNA is isolated from a mammalian, plant, bacterial, or fungal cell (e.g., gDNA).

In some embodiments, the DNA is synthetic.

In some embodiments, the engineered nuclease is a TALEN, zinc finger, meganuclease, megaTAL, FokI-dCas9 fusion or a Cas9 nuclease or Cpf1 nuclease, e.g., wild type or a variant thereof.

In some embodiments, wherein the engineered nuclease is a Cas9 nuclease, the method also includes utilizing a guide RNA that directs the Cas9 nuclease to a target sequence in the genome. In some embodiments, the engineered nuclease is a Cas9 nuclease, and the method also includes expressing in the cells a guide RNA that directs the Cas9 nuclease to a target sequence in the genome.

In some embodiments, the primer site in the hairpin comprises a next generation sequencing primer site, a randomized DNA barcode or unique molecular identifier (UMI).

The present methods have several advantages over previously described approaches for finding off-target sites of engineered nucleases. For example, the present methods are *in vitro*; in contrast, cell-based methods (such as GUIDE-seq (see WO 2015/200378 and Tsai et al., Nature Biotechnology 33:187–197 (2015)) require the introduction of double stranded oligodeoxynucleotides (dsODN) as well as expression or introduction of nuclease or nuclease-encoding components into cells. Not all cells will allow the introduction of dsODNs and/or nuclease or nuclease-encoding components, and these reagents can be toxic in some cases. If a cell type of

particular interest is not amenable to introduction of dsODNs, nucleases, or nuclease-encoding components, a surrogate cell type might be used, but cell-specific effects might not be detected. The present methods do not require delivery of dsODNs, nucleases, or nuclease-encoding components into a cell, are not influenced by chromatin state, do not create toxicity issues, do not require growth and propagation of a cell line, and enable interrogation of specific cellular genomes by cleavage of genomic DNA that is obtained from the cell-type of interest. Furthermore, because the exonuclease has activity against double-stranded linear DNA but essentially none against circular DNA, the background is very low providing a very high signal to noise ratio.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

Figures 1A-D. Overview of *in vitro* Digenome-seq and CIRCLE-seq methods for genome-wide detection of CRISPR-Cas9 nuclease off-target cleavage. (A) Schematic overview of the digested genome sequencing (**Digenome-seq**) method. Genomic DNA is cleaved with Cas9 nuclease *in vitro,* purified, sheared, and then ligated to adapters for whole genome sequencing. Candidate off-target sites with an enrichment of uniform start mapping positions are identified by a scoring algorithm. (B) Schematic overview of the circularization for *in vitro* reporting of cleavage effects by sequencing (**CIRCLE-seq**) method. Genomic DNA is sheared and circularized and undesired linear DNA molecules are degraded away by exonuclease treatment. Circular DNA molecules containing a Cas9 cleavage site (shaded) can be linearized with Cas9, releasing those DNA ends for adapter ligation, PCR

amplification, and paired-end high-throughput sequencing. Note how each pair of reads generated by Cas9 cleavage contains complete sequence information for a single off-target site. (C) Detailed schematic overview of exemplary CIRCLE-seq method. Genomic DNA is randomly sheared to an average of ~300 bp, end-repaired, A-tailed, and ligated to uracil-containing stem-loop adapters. Covalently closed DNA molecules with stem-loop adapters ligated to both ends are selected for by treatment with a mixture of Lambda exonuclease and E. coli Exonuclease I. 4 bp overhangs are released with a mixture of USER enzyme and T4 PNK, and DNA molecules are circularized at low concentrations favoring intramolecular ligation. Unwanted linear DNA is degraded with Plasmid-Safe ATP-dependent DNase. Circular DNA is treated with Cas9–gRNA complex and cleaved, linearized DNA is ligated to sequencing adapters and amplified for high throughput sequencing. (D) CIRCLE-seq read counts were highly reproducible between independent CIRCLE-seq experiments. Scatterplots of CIRCLE-seq read counts between two independent CIRCLE-seq libraries prepared from the same source of genomic DNA (human U2OS cells). CIRCLE-seq read counts were strongly correlated.

Figures 2A-D. Comparison of CIRCLE-seq with Digenome-seq. (A) Venn diagram showing intersections of off-target sites of Cas9 and a gRNA targeted against the *HBB* gene detected by CIRCLE-seq and Digenome-seq. (B) Barplot of Digenome-seq read counts at off-target cleavage positions found by CIRCLE-seq but not Digenome-seq for nuclease-treated (shaded bars) and control (open bars) HAP1 genomic DNA. (C) Plots comparing mapping of sequencing reads for CIRCLE-seq and Digenome-seq at the on-target site of a gRNA targeted to the *HBB* locus. Both nuclease-treated and control samples are shown. Thin grey line indicates expected cleavage site position; read coverage for forward reads is colored in black, and reverse reads in white. (D) CIRCLE-seq start mapping position plot at the on-target site for the HBB gRNA used in (C). (+) strand mapping reads are shown in white bars, (-) strand mapping reads are colored in shaded bars.

Figures 3A-E. Comparisons of CIRCLE-seq with cell-based GUIDE-seq and HTGTS methods. (A) Histogram showing the number of sites identified exclusively by CIRCLE-seq (white bars) and by both CIRCLE-seq and GUIDE-seq (shaded bars) (B) Manhattan plots of CIRCLE-seq detected off-target sites, with bar heights representing CIRCLE-seq read count (normalized to site with highest read count) and organized by chromosomal position. (C) Venn diagrams showing

intersection of CIRCLE-seq and GUIDE-seq detected genomic off-target cleavage sites. (D) Histogram showing the number of sites detected exclusively by CIRCLE-seq or by both CIRCLE-seq and HTGTS. (E) Venn diagrams showing overlap between sets of off-target cleavage sites detected between CIRCLE-seq, GUIDE-seq, and HTGTS. CIRCLE-seq detects virtually all off-target cleavage sites detected by both GUIDE-seq and HTGT).

Figures 4A-G. CIRCLE-seq detected off-target cleavage sites can also be cleaved in human cells. (A) Stem-leaf plot of CIRCLE-seq read counts for 10 gRNAs previously analyzed by GUIDE-seq. The on-target and off-target sites are shown. (B) Schematic overview of the targeted tag sequencing approach. Primers are designed to amplify genomic regions flanking nuclease-induced DSBs from genomic DNA of cells treated with nuclease and double-stranded oligodeoxynucleotide (dsODN) tag. (c-d) Targeted tag integration frequencies at control off-target sites detected by both CIRCLE-seq and GUIDE-seq (upper part of panel) and off-target sites detected by CIRCLE-seq but not GUIDE-seq) for gRNAs targeted to *EMX1* and *VEGFA* site 1. Off-target sites are ordered top to bottom by CIRCLE-seq read count with mismatches to the intended target sequence indicated. Observed tag integration frequencies observed for control and nuclease-treated cells are plotted on a log scale. (E) Pie charts showing fractions of CIRCLE-seq sites analyzed that are also detected by targeted tag sequencing. (F) Plots of integration positions observed by targeted tag sequencing. PAM bases are the last three nucleotides from the right. Integrations occur at positions proximal to the location of the predicted DSB (three base pairs away the PAM). (G) Percentage of unique cleavage sites that can be found using a reference-independent site discovery algorithm, for CIRCLE-seq experiments performed with gRNAs targeting non-repetitive sites in HEK293, K562, and U2OS genomic DNA.

Figures 5A-E. Using CIRCLE-seq to assess the impacts of personalized SNPs on off-target site analysis. (A) Scatterplots of CIRCLE-seq read counts from experiments performed on genomic DNA from two different cell types. Sites with non-reference genetic variation in only one cell type are black, while those with non-reference variation in both cell types are shaded. (B) Examples of allele-specific CIRCLE-seq read counts at off-target sites with non-reference genetic variation. Mismatches to the intended target sequence are indicated with colored nucleotides, while matching bases are indicated with a dot. The base position harboring the

differential genetic change between cell types is indicated with a small arrow. (C) Proportion of CIRCLE-seq off-target sites where non-reference genetic variation was identified in genotyped individuals from the 1000 Genomes Project: African (AFR), Ad Mixed American (AMR), East Asian (EAS), European (EUR), and Southeast Asian (SAS) superpopulations, and a combined population average. (D) Histogram showing distribution of CIRCLE-seq off-target sites by numbers of mismatches in reference human genome sequence (striped bars) and in 1000 Genomes Project data-derived off-target site haplotypes (white bars). (E) Proportion of 1000 Genomes Project-derived haplotypes with increased, decreased, or the same numbers of mismatches in off-target sites identified by CIRCLE-seq.

DETAILED DESCRIPTION

Engineered genome-editing nucleases are transformative technologies for both scientific research and human medicine. An important issue with the use of this technology is the extent to which off-target cleavage events occur at unintended target sites in the genome of a living cell.[10,24-27] This is significant particularly because, in nearly all cell types, repair of nuclease-induced breaks by non-homologous end-joining can lead to the efficient introduction of insertion or deletion mutations (indels). For both research and therapeutic applications, in which millions to billions of cells may be treated with these nucleases, even low-frequency mutations may lead to unwanted cellular phenotypes or undesired clinical consequences.

A number of genome-wide approaches have recently been developed to define the landscape of off-target cleavage events induced by genome-editing nucleases. For example, various cell-based methods for genome-wide detection of such mutations have been described.[28-32] These include Genome-wide Unbiased Identification of DSBs enabled by sequencing (GUIDE-seq) and High-throughput, Genome-wide Translocation Sequencing (HTGTS). For example, the GUIDE-seq method, which relies on uptake of a short double-stranded oligonucleotide "tag" into nuclease-induced DSBs in living cells, has been shown to define off-target sites on a genome-wide scale, identifying sites that are mutagenized with frequencies as low as 0.1% of the time in a population of cells (Tsai et al., Nat Biotechnol. 2015). Other cell-based methods for defining nuclease-induced off-target breaks include a method that maps translocation fusions to the on-target site and another that relies on uptake of integration-deficient lentivirus (IDLV) genomes into sites of DSBs. However, the

requirements of these methods for efficient manipulation of cells can limit their feasibility, scalability, and reproducibility, particularly when working with more challenging non-transformed cell types that would be most relevant and useful for therapeutics.

Despite this recent progress, cell-based methods for off-target determination have a number of limitations including: (1) a requirement to be able to introduce both the nuclease components and a tag such as the dsODN or IDLV genome into cells; (2) biological selection pressures that might favor or disfavor the growth of cells harboring certain types of off-target mutations; (3) the potential confounding effects of cell-type-specific parameters such as chromatin, DNA methylation, gene expression, and nuclear architecture on nuclease off-target activities/effects; and/or (4) the requirement to be able to grow the cells of interest in culture.

In vitro methods using purified genomic DNA provide an attractive alternative because they would sidestep these various limitations of cell-based approaches. (Note that in this description, in vitro refers to experiments performed with purified components in cell-free reactions). In vitro methods for detecting off-target cleavage sites have potential advantages over cell-based methods that can include: a) they are not affected by chromatin context, gene expression levels, or intra-nuclear localization because they are performed on purified protein-free DNA, b) they do not depend on error-prone cellular DNA repair for detection of sites, which can be influenced by the factors listed in a) as well as by sequence-dependent effects, and c) they offer the potential to be highly sensitive for detection of low frequency cleavage events because the concentrations of nuclease, genomic DNA, and length of time for cleavage can all be varied. Biochemical assays using defined and purified components improve reproducibility, bypass the need for efficient cell transduction or transfection, and avoid potential biases caused by positive or negative effects on cell fitness. Importantly, the concentrations of active nuclease and genomic DNA can be raised to very high levels *in vitro*, potentially enabling identification of sequences that may be cleaved at very rare frequencies in cells and/or at the highest concentrations of nucleases that can be expressed in cells. An *in vitro* method for characterizing Cas9 cleavage specificity of partially degenerate DNA libraries biased towards specific target DNA sites has been previously described but when used with Cas9 nuclease and various gRNAs, most of the sites identified did not actually occur in the human genome[33].

However, in vitro methods face the challenge that isolated genomic DNA is by experimental necessity randomly sheared (or broken) into smaller pieces. This poses a challenge because it is not easy to differentially identify DSBs induced by shearing from DSBs induced by treatment of the genomic DNA in vitro with nucleases. To date, only a single *in vitro* genome-wide off-target identification method, known as Digenome-seq[34], has been described for use with human genomic DNA. This approach relies on nuclease cleavage of bulk genomic DNA, ligation of sequencing adapters to all free ends (nuclease- and non-nuclease-induced), high-throughput sequencing, and bioinformatic identification of nuclease-cleaved sites with signature uniform mapping ends. However, the extremely high background of randomly sheared genomic DNA fragments sequenced with Digenome-seq makes it exceedingly challenging to identify lower frequency nuclease-induced cleavage events and requires access to HiSeq or HiSeq X10 machines used for production-scale human genome resequencing that can generate the required number of reads (>400 million).

The Digenome-seq method suffers from a high background of sequencing reads that are uninformative about nuclease off-target activity, making it inefficient with respect to sequencing resources and therefore less sensitive in its ability to detect off-target sites. With Digenome-seq, randomly sheared genomic DNA is subjected to digestion by an engineered nuclease and then these fragments are subjected to whole genome re-sequencing. Randomly sheared fragments will align randomly to the genome, whereas those fragments that have been digested by the nuclease will have uniform ends that when mapped back to the genome will line up at the same genomic base position. An initial validation of this method showed that it had some ability to detect off-target cleavage sites for CRISPR-Cas9 nucleases. However, with Digenome-seq, less than 1 in every million sequencing reads are mapped to a nuclease off-target site. This high background causes several disadvantages for the method: (A) it makes the method extremely cost-ineffective with respect to sequencing, requiring the use of methods such as the HiSeq or HiSeqx10 platform; (B) the high background of randomly broken DNA fragments that map to the genome make it challenging to identify low frequency nuclease-induced breaks, a signal-to-noise problem; and (C) the low yield of information makes it challenging to find low frequency nuclease off-target sites even with the large number of sequencing reads that can be obtained with state of the art methods such as the HiSeqx10 platform. Indeed, Digenome failed to

identify a number of off-target sites found by GUIDE-seq for a particular gRNA (although the caveat must be added that the two experiments were performed with genomic DNA from different cell lines).

Described herein is an *in vitro* method that enables comprehensive determination of DSBs induced by nucleases on any genomic DNA of interest. This method enables enrichment of nuclease-induced DSBs over random DSBs induced by shearing of genomic DNA.

An exemplary method, described herein as Circularization for In vitro Reporting of CLeavage Effects by sequencing (CIRCLE-seq), is a novel cell-free strategy that enables highly efficient selective enrichment of nuclease-cleaved genomic DNA, which can then be used for high-throughput sequence and genomic off-target cleavage site discovery. Similar to the Full Interrogation of Nuclease DSBs by sequencing (FIND-seq) method (described in USSN 62/217,690), CIRCLE-seq is based on the principle of generating a starting library of randomly sheared genomic DNA fragments whose DNA ends are protected in a way that prevents any subsequent ligation of a sequencing adapter. However, in CIRCLE-seq, this population of molecules is created by a novel unbiased intramolecular circularization method followed by exonuclease treatment to degrade any remaining residual linear fragments (Figure 2). This enzymatically purified population of covalently closed circular genomic DNA molecules is then treated with an engineered nuclease of choice. Any DNA fragments harboring sites cleaved by this nuclease will be linearized, thereby releasing two available DNA ends to which a sequencing adapter can be ligated. Linearized, adapter-ligated fragments are subsequently amplified by PCR for high-throughput sequencing (See Figure 2). Thus, CIRCLE-Seq virtually eliminates the very high background of random genomic reads observed with Digenome-seq.

In vitro conditions for the efficient intramolecular circularization of gDNA molecules at low concentrations of DNA were optimized, as this process is critical for the success and low background of this method. CIRCLE-seq enrichment of nuclease-cleaved fragments (based on experiments with CRISPR-Cas9 nucleases) was estimated to be nearly 6 orders of magnitude above background, thereby substantially reducing the high background of randomly sheared DNA fragments that are sequenced with a brute-force *in vitro* off-target cleavage site discovery method like Digenome-seq (**Fig. 1A**). The enrichment factor was calculated at the on-target site (where nearly 100% of fragments are expected to be cleaved *in vitro*) by dividing the

number of reads that originate from the predicted breakpoint by the number of reads at those positions that would be expected by chance at the sequencing depth of the experiment.

Importantly, reducing the background dramatically increased the signal-to-noise ratio resulting from the CIRCLE-seq method, and reduced the probability of false positive calls due to the recovery of reads with uniform ends that can occur by chance with Digenome-seq. Initial circularization of genomic DNA fragments also enables the simultaneous recovery of both ends of a DSB in a single DNA molecule (and therefore a single DNA sequencing read), which in turns enables reference-independent discovery of off-target cleavage sites, an improvement over all previously described method for identifying nuclease-induced off-target effects that enables the approach to be used with genomic DNA from any organism, regardless of whether its genome sequence is known in advance. In addition, because there is minimal processing of the ends *in vitro* (as opposed to in cells where DNA repair pathways can resect or degrade ends), this method also greatly improves the nucleotide-level precision with which one can assign the location of the nuclease-induced break.

In initial validation experiments of CIRCLE-seq using CRISPR-Cas9 nucleases, the enrichment for Cas9 nuclease-cleaved fragments was nearly 20-fold more for CIRCLE-seq compared to the FIND-seq method (described in USSN 62/217,690 and Fig. 1). A fundamental difference between these two methods is that FIND-seq is based on linear DNA substrate that is covalently closed with stem-loop adapters, whereas CIRCLE-seq is based on covalently closed fully circularized DNA substrates (**Fig. 1A**). One of the preferred exonucleases (the commercially-available PlasmidSafe ATP-dependent DNase from Epicentre) may retain some activity against the exposed ssDNA portions of the FIND-seq stem-loop ligated DNA substrates, which may explain why the CIRCLE-seq method results in higher enzymatic discrimination between linear and covalently closed DNA fragments and lower overall background.

Thus, the results provided show that CIRCLE-seq method is the most sensitive and sequencing-efficient *in vitro* approach described to date for determining genome-wide off-target cleavage sites of CRISPR-Cas9 nucleases. CIRCLE-seq has a substantially reduced rate of observed background reads relative to Digenome-seq, enabling it to sensitively identify off-target sites using a small fraction (~1.7%) of the

total number of sequencing reads required with Digenome-seq. This need for only a more modest number of reads (e.g., those obtained with a benchtop sequencing instrument such as the Illumina MiSeq) makes the method accessible to most labs and more amenable from a cost perspective to automation and scaling.

CIRCLE-seq enables the production of larger datasets that permit the training of more accurate predictive algorithms for Cas9 off-target cleavage. The method can be cost-effectively automated and scaled and therefore it should be feasible to produce data identifying the off-target cleavage sites and relative *in vitro* cleavage efficiencies (as measured by CIRCLE-seq read counts) of thousands of gRNAs. Furthermore, if coupled with large-scale cell-based off-target datasets determined in ENCODE-characterized cell lines[39] with methods such as GUIDE-seq, it may be possible to better understand and quantify the impacts of chromatin and epigenetic modifications on the ability of nucleases to induce DNA DSBs.

For therapeutic applications, because CIRCLE-seq outperforms even the most highly sensitive cell-based genome-wide off-target detection methods such as GUIDE-seq and HTGTS, the method could be used as an initial screen to identify potential off-target sites that can then be verified with an orthogonal approach in actual nuclease-modified cells. In this study, targeted sequencing was used to search for GUIDE-seq dsODN tags to validate low-frequency sites (<0.1%) that would be challenging to identify by standard amplicon sequencing due to the indel error rate associated with next-generation sequencing (typically ~0.1%). However, this approach is limited to cells that can be transfected with the GUIDE-seq dsODN tag, something that may not be possible to do for all cells. Thus, an important and urgent area for future studies will be the development of alternative orthogonal cell-based methods to more sensitively measure off-target mutagenesis below the error rate of current high-throughput sequencing technologies. CIRCLE-seq can also be extended to analyze off-target effects of other nuclease platforms already in use for human therapeutics such as engineered zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs).

The CIRCLE-seq data herein provide greater support for the potential impacts of human genetic variation on off-target cleavage, a concept initially raised by others[36]. Our findings reinforce the importance of considering genotypes when evaluating off-target risk and argue that safety assessments of nucleases should include specificity profiles performed in a patient-specific way. Alternatively,

CIRCLE-seq performed on a genomic DNAs from a large panel of genetically diverse cells may provide an effective way to define the vast majority of common and SNP-specific off-target effects for any given nuclease. In this regard, CIRCLE-seq has been performed on genomic DNA isolated from primary fibroblasts as would be obtained from a skin biopsy and buffy coat from a standard blood draw would also provide ample amounts of DNA to perform the assay. The simplicity, scalability, and reproducibility of CIRCLE-seq makes it ideally suited for defining genome-wide off-target profiles on a large series of genomic DNA samples.

Stem-Loop Adapters and Sequencing Adapters

The present methods include the use of non-naturally occurring stem-loop adapters for circularizing the fragments by intramolecular ligation. The stem loop adapters include (from 5' to 3') a first region, e.g., of about 10-15, e.g., 12, nucleotides; a second region, e.g., of about 4-6, e.g., 5, nucleotides that forms at least one (and preferably only one) hairpin loops and includes a palindromic sequence suitable for intramolecular ligation, flanked by at least one (and preferably only one) uracil; and a third region, e.g., of about 10-15, e.g., 13, nucleotides that is complementary to the first region.

The present methods also include the use of sequencing adapters, which include a sequence of nucleotides for use in priming PCR or sequencing. The sequencing adapters typically include (from 5' to 3') a first region, e.g., of about 10-15, e.g., 12, nucleotides; a second region, e.g., of about 20-60, e.g., 40, nucleotides that forms at least one (and preferably only one) hairpin loops and includes a sequence suitable for use in PCR priming and/or sequencing, e.g., next generation sequencing (NGS), flanked by at least one (and preferably only one) uracil; and a third region, e.g., of about 10-15, e.g., 13, nucleotides that is complementary to the first region. The lengths of the first, second and third regions can vary depending on the NGS method selected, as they are dependent on the sequences that are necessary for priming for use with the selected NGS platform. In some embodiments, commercially available adapters that are variations of standard adapters (e.g., from Illumina or NEB) can be used.

The stem loop and sequencing adapters include at least one, preferably only one, uracil that allows the adaptor to be opened by Uracil DNA glycosylase (UDG) and Endonuclease VIII, a DNA glycosylase-lyase, e.g., the USER (Uracil-Specific

Excision Reagent) Enzyme mixture (New England BioLabs). The UDG catalyzes the excision of uracil bases to form an abasic site but leave the phosphodiester backbone intact (see, e.g., Lindhal et al., J. Biol. Chem.. 252:3286-3294 (1977); Lindhal, Annu. Rev. Biochem. 51:61-64 (1982)). The Endonuclease VIII breaks the phosphodiester backbone at the 3′ and 5′ sides of the abasic site (see, e.g., Melamede et al., Biochemistry 33:1255-1264 (1994); Jiang et al., J. Biol. Chem. 272:32230-32239 (1997)). This combination generates a single nucleotide gap at the location of a uracil. In some embodiments, the uracil is placed at or within 1, 2, 3, or 4 nucleotides of the 3' or 5' end of the sequence compatible for use in PCR priming and/or sequencing or the palindromic sequence.

In the present methods, all parts of the adapters are preferably orthogonal to the genome of the cell (i.e., are not present in or complementary to a sequence present in, i.e., have no more than 10%, 20%, 30%, 40%, or 50% identity to a sequence present in, the genome of the cell).

The sequencing adapters should preferably include a sequence compatible for use in PCR priming and/or sequencing, e.g., a primer site that is a randomized DNA barcode (e.g., SHAPE-SEQ, Lucks et al., Proc Natl Acad Sci U S A 108: 11063-11068), unique molecular identifier (UMI) (see, e.g., Kivioja et al., Nature Methods 9, 72–74 (2012); Islam et al., Nature Methods 11, 163–166 (2014); Karlsson et al., Genomics. 2015 Mar;105(3):150-8), or unique PCR priming sequence and/or unique sequence compatible for use in sequencing (e.g., NGS). The sequence compatible for use in sequencing can be selected for use with a desired sequencing method, e.g., a next generation sequencing method, e.g., Illumina, Ion Torrent or library preparation method like Roche/454, lllumina Solexa Genome Analyzer, the Applied Biosystems SOLiD™ system, Ion Torrent™ semiconductor sequence analyzer, PacBio® real-time sequencing and Helicos™ Single Molecule Sequencing (SMS). See, e.g., WO2014020137, Voelkerding et al., Clinical Chemistry 55:4 641-658 (2009) and Metzker, Nature Reviews Genetics 11:31-46 (2010)). A number of kits are commercially available for preparing DNA for NGS, including the ThruPLEX DNA-seq Kit (Rubicon; see U.S. Patents 7,803,550; 8,071,312; 8,399,199; 8,728,737) and NEBNext® (New England BioLabs; see e.g., U.S. patent 8,420,319)). An exemplary stem loop adapter sequence is /5Phos/CGGTGGACCGATGATC /ideoxyU/ ATCGGTCCACCG*T (SEQ ID NO:1; the asterisk indicates a phosphorothioate

linkage). In some embodiments, the sequencing adapter is a commercially available sequencing adapter, e.g., from the NEBNext kit.

In some embodiments, the adapters include a restriction enzyme recognition site, preferably a site that is relatively uncommon in the genome of the cell.

The adapters are preferably modified; in some embodiments, the 5' ends of the hairpin adapters are phosphorylated, and/or the 3' ends include a phosphorothioate linkage. In some embodiments, the adapters are blunt ended. In some embodiments, the adapters include a random variety of 1, 2, 3, 4 or more nucleotide overhangs on the 5' or 3' ends, or include a single T at the 5' or 3' end.

The adapters can also include one or more additional modifications, e.g., as known in the art or described in PCT/US2011/060493. For example, in some embodiments, the hairpin adapters is biotinylated. The biotin can be anywhere internal to the hairpin adapters (e.g., a modified thymidine residue (Biotin-dT) or using biotin azide), but not on the 5' or 3' ends. This provides an alternate method of recovering fragments that contain ligated sequencing adapters. Whereas in some embodiments, these sequences are retrieved and amplified by PCR, in this approach they are physically pulled down and enriched by using the biotin, e.g., by binding to streptavidin-coated magnetic beads, or using solution hybrid capture; see, e.g., Gnirke et al., Nature Biotechnology 27, 182 - 189 (2009).

Engineered Nucleases

There are presently four main classes of engineered nucleases: 1) meganucleases, 2) zinc-finger nucleases, 3) transcription activator effector-like nucleases (TALEN), and 4) Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas RNA-guided nucleases (RGN). Various components of these platforms can also be fused together to create additional nucleases such as Mega-TALs and FokI-dCas9 fusions. See, e.g., Gaj et al., Trends Biotechnol. 2013 Jul;31(7):397-405. The nuclease can be transiently or stably expressed in the cell, using methods known in the art; typically, to obtain expression, a sequence encoding a protein is subcloned into an expression vector that contains a promoter to direct transcription. Suitable eukaryotic expression systems are well known in the art and described, e.g., in Sambrook et al., *Molecular Cloning, A Laboratory Manual* (4th ed. 2013); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (2006); and *Current Protocols in Molecular Biology* (Ausubel et al., eds., 2010). Transformation

of eukaryotic and prokaryotic cells are performed according to standard techniques (see, e.g., the reference above and Morrison, 1977, J. Bacteriol. 132:349-351; Clark-Curtiss & Curtiss, Methods in Enzymology 101:347-362 (Wu et al., eds, 1983).

Homing Meganucleases

Meganucleases are sequence-specific endonucleases originating from a variety of organisms such as bacteria, yeast, algae and plant organelles. Endogenous meganucleases have recognition sites of 12 to 30 base pairs; customized DNA binding sites with 18bp and 24bp-long meganuclease recognition sites have been described, and either can be used in the present methods and constructs. See, e.g., Silva, G., et al., Current Gene Therapy, 11:11-27, (2011); Arnould et al., Journal of Molecular Biology, 355:443-58 (2006); Arnould et al., Protein Engineering Design & Selection, 24:27-31 (2011); and Stoddard, Q. Rev. Biophys. 38, 49 (2005); Grizot et al., Nucleic Acids Research, 38:2006-18 (2010).

CRISPR-Cas Nucleases

Recent work has demonstrated that clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems (Wiedenheft et al., Nature 482, 331-338 (2012); Horvath et al., Science 327, 167-170 (2010); Terns et al., Curr Opin Microbiol 14, 321-327 (2011)) can serve as the basis of a simple and highly efficient method for performing genome editing in bacteria, yeast and human cells, as well as *in vivo* in whole organisms such as fruit flies, zebrafish and mice (Wang et al., Cell 153, 910-918 (2013); Shen et al., Cell Res (2013); Dicarlo et al., Nucleic Acids Res (2013); Jiang et al., Nat Biotechnol 31, 233-239 (2013); Jinek et al., Elife 2, e00471 (2013); Hwang et al., Nat Biotechnol 31, 227-229 (2013); Cong et al., Science 339, 819-823 (2013); Mali et al., Science 339, 823-826 (2013c); Cho et al., Nat Biotechnol 31, 230-232 (2013); Gratz et al., Genetics 194(4):1029-35 (2013)). The Cas9 nuclease from *S. pyogenes* (hereafter simply Cas9) can be guided via simple base pair complementarity between 17-20 nucleotides of an engineered guide RNA (gRNA), e.g., a single guide RNA or crRNA/tracrRNA pair, and the complementary strand of a target genomic DNA sequence of interest that lies next to a protospacer adjacent motif (PAM), e.g., a PAM matching the sequence NGG or NAG (Shen et al., Cell Res (2013); Dicarlo et al., Nucleic Acids Res (2013); Jiang et al., Nat Biotechnol 31, 233-239 (2013); Jinek et al., Elife 2, e00471 (2013); Hwang et al., Nat Biotechnol 31, 227-229 (2013); Cong et al., Science 339, 819-823 (2013); Mali et al., Science 339, 823-826 (2013c); Cho et al., Nat Biotechnol 31, 230-232 (2013); Jinek et al.,

Science 337, 816-821 (2012)). The engineered CRISPR from Prevotella and Francisella 1 (Cpf1) nuclease can also be used, e.g., as described in Zetsche et al., Cell 163, 759-771 (2015); Schunder et al., Int J Med Microbiol 303, 51-60 (2013); Makarova et al., Nat Rev Microbiol 13, 722-736 (2015); Fagerlund et al., Genome Biol 16, 251 (2015). Unlike SpCas9, Cpf1 requires only a single 42-nt crRNA, which has 23 nt at its 3' end that are complementary to the protospacer of the target DNA sequence (Zetsche et al., 2015). Furthermore, whereas SpCas9 recognizes an NGG PAM sequence that is 3' of the protospacer, AsCpf1 and LbCp1 recognize TTTN PAMs that are found 5' of the protospacer (*Id.*).

In some embodiments, the present system utilizes a wild type or variant Cas9 protein from *S. pyogenes* or *Staphylococcus aureus*, or a wild type Cpf1 protein from *Acidaminococcus sp. BV3L6* or *Lachnospiraceae bacterium ND2006* either as encoded in bacteria or codon-optimized for expression in mammalian cells and/or modified in its PAM recognition specificity and/or its genome-wide specificity. A number of variants have been described; see, e.g., WO 2016/141224, PCT/US2016/049147, Kleinstiver et al., Nat Biotechnol. 2016 Aug;34(8):869-74; Tsai and Joung, Nat Rev Genet. 2016 May;17(5):300-12; Kleinstiver et al., Nature. 2016 Jan 28;529(7587):490-5; Shmakov et al., Mol Cell. 2015 Nov 5;60(3):385-97; Kleinstiver et al., Nat Biotechnol. 2015 Dec;33(12):1293-1298; Dahlman et al., Nat Biotechnol. 2015 Nov;33(11):1159-61; Kleinstiver et al., Nature. 2015 Jul 23;523(7561):481-5; Wyvekens et al., Hum Gene Ther. 2015 Jul;26(7):425-31; Hwang et al., Methods Mol Biol. 2015;1311:317-34; Osborn et al., Hum Gene Ther. 2015 Feb;26(2):114-26; Konermann et al., Nature. 2015 Jan 29;517(7536):583-8; Fu et al., Methods Enzymol. 2014;546:21-45; and Tsai et al., Nat Biotechnol. 2014 Jun;32(6):569-76, *inter alia*. The guide RNA is expressed or present in the cell together with the Cas9 or Cpf1. Either the guide RNA or the nuclease, or both, can be expressed transiently or stably in the cell or introduced as a purified protein or nucleic acid.

In some embodiments, the nuclease is a FokI-dCas9 fusion, RNA-guided FokI nucleases in which Cas9 nuclease has been rendered catalytically inactive by mutation (e.g., dCas9) and a FokI nuclease fused in frame, optionally with an intervening linker, to the dCas9. See, e.g., WO 2014/144288 and WO 2014/204578.

TAL Effector Repeat Arrays

TAL effectors of plant pathogenic bacteria in the genus Xanthomonas play important roles in disease, or trigger defense, by binding host DNA and activating effector-specific host genes. Specificity depends on an effector-variable number of imperfect, typically ~33-35 amino acid repeats. Polymorphisms are present primarily at repeat positions 12 and 13, which are referred to herein as the repeat variable-diresidue (RVD). The RVDs of TAL effectors correspond to the nucleotides in their target sites in a direct, linear fashion, one RVD to one nucleotide, with some degeneracy and no apparent context dependence. In some embodiments, the polymorphic region that grants nucleotide specificity may be expressed as a triresidue or triplet.

Each DNA binding repeat can include a RVD that determines recognition of a base pair in the target DNA sequence, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA sequence. In some embodiments, the RVD can comprise one or more of: HA for recognizing C; ND for recognizing C; HI for recognizing C; HN for recognizing G; NA for recognizing G; SN for recognizing G or A; YG for recognizing T; and NK for recognizing G, and one or more of: HD for recognizing C; NG for recognizing T; NI for recognizing A; NN for recognizing G or A; NS for recognizing A or C or G or T; N* for recognizing C or T, wherein * represents a gap in the second position of the RVD; HG for recognizing T; H* for recognizing T, wherein * represents a gap in the second position of the RVD; and IG for recognizing T.

TALE proteins may be useful in research and biotechnology as targeted chimeric nucleases that can facilitate homologous recombination in genome engineering (e.g., to add or enhance traits useful for biofuels or biorenewables in plants). These proteins also may be useful as, for example, transcription factors, and especially for therapeutic applications requiring a very high level of specificity such as therapeutics against pathogens (e.g., viruses) as non-limiting examples.

Methods for generating engineered TALE arrays are known in the art, see, e.g., the fast ligation-based automatable solid-phase high-throughput (FLASH) system described in USSN 61/610,212, and Reyon et al., Nature Biotechnology 30,460–465 (2012); as well as the methods described in Bogdanove & Voytas, Science 333, 1843-1846 (2011); Bogdanove et al., Curr Opin Plant Biol 13, 394-401 (2010); Scholze & Boch, J. Curr Opin Microbiol (2011); Boch et al., Science 326, 1509-1512 (2009);

Moscou & Bogdanove, Science 326, 1501 (2009); Miller et al., Nat Biotechnol 29, 143-148 (2011); Morbitzer et al., T. Proc Natl Acad Sci U S A 107, 21617-21622 (2010); Morbitzer et al., Nucleic Acids Res 39, 5790-5799 (2011); Zhang et al., Nat Biotechnol 29, 149-153 (2011); Geissler et al., PLoS ONE 6, e19509 (2011); Weber et al., PLoS ONE 6, e19722 (2011); Christian et al., Genetics 186, 757-761 (2010); Li et al., Nucleic Acids Res 39, 359-372 (2011); Mahfouz et al., Proc Natl Acad Sci U S A 108, 2623-2628 (2011); Mussolino et al., Nucleic Acids Res (2011); Li et al., Nucleic Acids Res 39, 6315-6325 (2011); Cermak et al., Nucleic Acids Res 39, e82 (2011); Wood et al., Science 333, 307 (2011); Hockemeye et al. Nat Biotechnol 29, 731-734 (2011); Tesson et al., Nat Biotechnol 29, 695-696 (2011); Sander et al., Nat Biotechnol 29, 697-698 (2011); Huang et al., Nat Biotechnol 29, 699-700 (2011); and Zhang et al., Nat Biotechnol 29, 149-153 (2011); all of which are incorporated herein by reference in their entirety.

Also suitable for use in the present methods are MegaTALs, which are a fusion of a meganuclease with a TAL effector; see, e.g., Boissel et al., Nucl. Acids Res. 42(4):2591-2601 (2014); Boissel and Scharenberg, Methods Mol Biol. 2015;1239:171-96.

Zinc Fingers

Zinc finger proteins are DNA-binding proteins that contain one or more zinc fingers, independently folded zinc-containing mini-domains, the structure of which is well known in the art and defined in, for example, Miller et al., 1985, EMBO J., 4:1609; Berg, 1988, Proc. Natl. Acad. Sci. USA, 85:99; Lee et al., 1989, Science. 245:635; and Klug, 1993, Gene, 135:83. Crystal structures of the zinc finger protein Zif268 and its variants bound to DNA show a semi-conserved pattern of interactions, in which typically three amino acids from the alpha-helix of the zinc finger contact three adjacent base pairs or a "subsite" in the DNA (Pavletich et al., 1991, Science, 252:809; Elrod-Erickson et al., 1998, Structure, 6:451). Thus, the crystal structure of Zif268 suggested that zinc finger DNA-binding domains might function in a modular manner with a one-to-one interaction between a zinc finger and a three-base-pair "subsite" in the DNA sequence. In naturally occurring zinc finger transcription factors, multiple zinc fingers are typically linked together in a tandem array to achieve sequence-specific recognition of a contiguous DNA sequence (Klug, 1993, Gene 135:83).

Multiple studies have shown that it is possible to artificially engineer the DNA binding characteristics of individual zinc fingers by randomizing the amino acids at the alpha-helical positions involved in DNA binding and using selection methodologies such as phage display to identify desired variants capable of binding to DNA target sites of interest (Rebar et al., 1994, Science, 263:671; Choo et al., 1994 Proc. Natl. Acad. Sci. USA, 91:11163; Jamieson et al., 1994, Biochemistry 33:5689; Wu et al., 1995 Proc. Natl. Acad. Sci. USA, 92: 344). Such recombinant zinc finger proteins can be fused to functional domains, such as transcriptional activators, transcriptional repressors, methylation domains, and nucleases to regulate gene expression, alter DNA methylation, and introduce targeted alterations into genomes of model organisms, plants, and human cells (Carroll, 2008, Gene Ther., 15:1463-68; Cathomen, 2008, Mol. Ther., 16:1200-07; Wu et al., 2007, Cell. Mol. Life Sci., 64:2933-44).

One existing method for engineering zinc finger arrays, known as "modular assembly," advocates the simple joining together of pre-selected zinc finger modules into arrays (Segal et al., 2003, Biochemistry, 42:2137-48; Beerli et al., 2002, Nat. Biotechnol., 20:135-141; Mandell et al., 2006, Nucleic Acids Res., 34:W516-523; Carroll et al., 2006, Nat. Protoc. 1:1329-41; Liu et al., 2002, J. Biol. Chem., 277:3850-56; Bae et al., 2003, Nat. Biotechnol., 21:275-280; Wright et al., 2006, Nat. Protoc., 1:1637-52). Although straightforward enough to be practiced by any researcher, recent reports have demonstrated a high failure rate for this method, particularly in the context of zinc finger nucleases (Ramirez et al., 2008, Nat. Methods, 5:374-375; Kim et al., 2009, Genome Res. 19:1279-88), a limitation that typically necessitates the construction and cell-based testing of very large numbers of zinc finger proteins for any given target gene (Kim et al., 2009, Genome Res. 19:1279-88).

Combinatorial selection-based methods that identify zinc finger arrays from randomized libraries have been shown to have higher success rates than modular assembly (Maeder et al., 2008, Mol. Cell, 31:294-301; Joung et al., 2010, Nat. Methods, 7:91-92; Isalan et al., 2001, Nat. Biotechnol., 19:656-660). In preferred embodiments, the zinc finger arrays are described in, or are generated as described in, WO 2011/017293 and WO 2004/099366. Additional suitable zinc finger DBDs are described in U.S. Pat. Nos. 6,511,808, 6,013,453, 6,007,988, and 6,503,717 and U.S. patent application 2002/0160940.

DNA

The methods described herein can be applied to DNA, e.g., genomic DNA isolated from any cell, artificially created populations of DNAs, or any other DNA pools, as it is performed *in vitro*.

Sequencing

As used herein, "sequencing" includes any method of determining the sequence of a nucleic acid. Any method of sequencing can be used in the present methods, including chain terminator (Sanger) sequencing and dye terminator sequencing. In preferred embodiments, Next Generation Sequencing (NGS), a high-throughput sequencing technology that performs thousands or millions of sequencing reactions in parallel, is used. Although the different NGS platforms use varying assay chemistries, they all generate sequence data from a large number of sequencing reactions run simultaneously on a large number of templates. Typically, the sequence data is collected using a scanner, and then assembled and analyzed bioinformatically. Thus, the sequencing reactions are performed, read, assembled, and analyzed in parallel; see, e.g., US 20140162897, as well as Voelkerding et al., Clinical Chem., 55: 641-658, 2009; and MacLean et al., Nature Rev. Microbiol., 7: 287-296 (2009). Some NGS methods require template amplification and some that do not. Amplification-requiring methods include pyrosequencing (see, e.g., U.S. Pat. Nos. 6,210,89 and 6,258,568; commercialized by Roche); the Solexa/Illumina platform (see, e.g., U.S. Pat. Nos. 6,833,246, 7,115,400, and 6,969,488); and the Supported Oligonucleotide Ligation and Detection (SOLiD) platform (Applied Biosystems; see, e.g., U.S. Pat. Nos. 5,912,148 and 6,130,073). Methods that do not require amplification, e.g., single-molecule sequencing methods, include nanopore sequencing, HeliScope (U.S. Pat. Nos. 7,169,560; 7,282,337; 7,482,120; 7,501,245; 6,818,395; 6,911,345; and 7,501,245); real-time sequencing by synthesis (see, e.g., U.S. Pat. No. 7,329,492); single molecule real time (SMRT) DNA sequencing methods using zero-mode waveguides (ZMWs); and other methods, including those described in U.S. Pat. Nos. 7,170,050; 7,302,146; 7,313,308; and 7,476,503). See, e.g., US 20130274147; US20140038831; Metzker, Nat Rev Genet 11(1): 31-46 (2010).

Alternatively, hybridization-based sequence methods or other high-throughput methods can also be used, e.g., microarray analysis, NANOSTRING, ILLUMINA, or other sequencing platforms.

Kits

Also provided herein are kits for use in the methods described herein. The kits can include one or more of the following: hairpin adapters; reagents and/or enzymes for end repair and A tailing (e.g., T4 polymerase, Klenow fragment, T4 Polynucleotide Kinase (PNK), and/or Taq DNA Polymerase); exonuclease; uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase, e.g., the USER (Uracil-Specific Excision Reagent) Enzyme mixture (New England BioLabs); purified nuclease, e.g., cas9 protein; guideRNA (e.g., control gRNA); gDNA template (e.g., control gDNA template); and/or instructions for use in a method described herein.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Materials and Methods

The following materials and methods were used in Examples 1-6 below.

Cell culture and transfection

Cell culture experiments were performed on human U2OS (gift from T. Cathomen), HEK293 (Thermo-Fisher), K562, and PGP1 fibroblast cells (gift from G. Church). U2OS and HEK293 cells were cultured in Advanced DMEM (Life Technologies) supplemented with 10% FBS, 2 mM GlutaMax (Life Technologies) and penicillin/streptomycin at 37°C with 5% CO_2. K562 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 2 mM GlutaMax and penicillin/streptomycin at 37°C with 5% CO_2. Human PGP1 fibroblasts were cultured in Eagle's DMEM (ATCC) with 10% FBS, 2 mM GlutaMax and penicillin/streptomycin at 37°C with 5% CO_2. For CIRCLE-seq experiments, genomic DNA was isolated using Gentra Puregene Tissue Kit (Qiagen) and quantified by Qubit (Thermo Fisher). For targeted tag-integration deep-sequencing experiments, U2OS cells (program DN-100), HEK293 cells (program CM-137), and K562 cells (program FF-120) were transfected in 20 µl Solution SE (Lonza) on a Lonza

Nucleofector 4-D, according to the manufacturer's instructions. In U2OS cells, 500 ng of pCAG-Cas9 (pSQT817), 250 ng of gRNA encoding plasmids, and 100 pmol of GUIDE-seq end-protected dsODN were cotransfected. Genomic DNA for targeted tag integration sequencing was harvested approximately 72 hours post transfection using the Agencourt DNAdvanced Genomic DNA Isolation Kit (Beckman Coulter Genomics).

***In vitro* transcription of gRNAs**

Annealed oligonucleotides containing gRNA target sites were cloned into plasmid NW59 containing a T7 RNA polymerase promoter site. The gRNA expression plasmid was linearized with HindIII restriction enzyme (NEB) and purified with MinElute PCR Purification Kit (Qiagen). The linearized plasmid was used as DNA template for *in vitro* transcription of the gRNA using MEGAshortscript Kit, according to the manufacturer's instructions (Thermo Fisher).

CIRCLE-seq library preparation

For the experiments with gRNAs previously evaluated by GUIDE-seq, CIRCLE-seq experiments were performed on genomic DNA from the same cells in which they were evaluated by GUIDE-seq (either U2OS or HEK293 cells). Purified genomic DNA was sheared with a Covaris S200 instrument to an average length of 300 bp, end-repaired, A-tailed, and ligated to uracil (deoxyuridine)-containing stem-loop adapter oSQT1288 5'-P-CGGTGGACCGATGATCUATCGGTCCACCG*T-3' (SEQ ID NO:1), where * indicates phosphorothioate linkage. Adapter-ligated DNA was treated with a mixture of Lambda Exonuclease (NEB) and *E.* coli Exonuclease I (NEB), then with USER enzyme (NEB) and T4 polynucleotide kinase (NEB). DNA was circularized at 5 ng/ul concentration with T4 DNA ligase, and treated with Plasmid-Safe ATP-dependent DNase (Epicentre) to degrade remaining linear DNA molecules. *In vitro* cleavage reactions were performed in a 100 µl, with Cas9 nuclease buffer (NEB), 90 nM SpCas9 protein, 90 nM *in vitro* transcribed gRNA, and 250 ng of Plasmid-Safe-treated circularized DNA. Digested products were A-tailed, ligated with a hairpin adapter, treated with USER enzyme (NEB), and amplified by PCR using Kapa HiFi polymerase (Kapa Biosystems). Completed libraries were quantified by droplet digital PCR (Bio-Rad) and sequenced with 150 bp paired end reads on an Illumina MiSeq instrument. Detailed user protocols for CIRCLE-seq library construction is provided in Example 7.

Targeted deep-sequencing

U2OS cells were transfected with Cas9 and gRNA expression plasmids, in addition to the GUIDE-seq dsODN as described above. Off-targets sites identified by CIRCLE-seq were amplified from the isolated U2OS genomic DNA using Phusion Hot Start Flex DNA polymerase (New England Biolabs). Triplicates of PCR products were generated from each transfection condition with 100 ng of genomic DNA as the input for each PCR. PCR products were normalized in concentration, pooled into different libraries corresponding to different transfection conditions, and purified with Ampure XP magnetic beads (Agencourt). Illumina Tru-seq deep-sequencing libraries were constructed using 500 ng of each pooled samples (KAPA Biosystems), quantified by real-time PCR (KAPA Biosystems), and sequenced on an Illumina MiSeq instrument.

CIRCLE-seq data analysis

Paired-end reads were merged and then mapped using *bwa*[40] *mem* with default parameters. The start mapping positions of reads that map in the expected orientation with mapping quality ≥ 50 were tabulated and genomic intervals that are enriched in nuclease-treated samples were identified. The interval and 20-bp of flanking reference sequence on either side was searched for potential nuclease-induced off-target sites with an edit distance of less than or equal to 6, allowing for gaps.

samtools[41,42] *mpileup* was used to non-reference genetic variation in identified off-target sites. Positions with average quality score greater than 20 were considered as possible variants and confirmed by visual inspection.

Reference-independent discovery of off-target cleavage sites was performed by reverse complementing the sequence of one read of a pair and concatenating it with the other. An interval of starting 20-bp on either side of the junction was directly searched for potential off-target cleavage sites with edit distance of ≤ 6 allowing for gaps and read counts corresponding to identified sites were tabulated.

CIRCLE-seq open-source analysis software

To enable the broad use of CIRCLE-seq for genome-wide detection of nuclease off-target sites, we developed a freely available, open-source Python package *circleseq* for the analysis of CIRCLE-seq experimental data. Provide with a simple sample manifest, the *circleseq* software performs full end-to-end analysis of CIRCLE-seq sequencing data with a single command, and returns tables of candidate off-target cleavage site positions, as well as visual alignments of off-target sequences.

Digenome-seq data analysis

Read counts of mapping positions in a narrow window (+/- 3 bp) around cleavage sites identified by CIRCLE-seq were tabulated from original Digenome-seq sequencing alignments. Significant evidence of cleavage at a 0.01 significance level was evaluated by fitting a negative binomial distribution, and statistically significant sites by this criteria were included in **Fig. 2b** .

Example 1. Overview and optimization of the CIRCLE-seq method

We reasoned that the confounding background reads of random genomic DNA that occur with Digenome-seq **(Fig. 1A)** would be substantially reduced by the selective enrichment of Cas9 nuclease-cleaved genomic DNA fragments. To accomplish this, we designed a restriction enzyme-independent strategy to circularize randomly sheared linear genomic DNA with subsequent enzymatic degradation of residual uncircularized DNA **(Figs. 1B-C)**. We envisioned that cleavage of this population of genomic DNA circles by a site-specific nuclease at on- and off-target sites would release linearized DNA ends, to which next-generation sequencing adapters could be ligated. We hypothesized that collectively these steps should enable the selective enrichment and sequencing of nuclease-induced DNA ends and suppress the undesired sequencing of randomly distributed ends sheared genomic DNA preparations. Importantly, in contrast to all other genome-wide methods for nuclease off-target cleavage site discovery, our strategy would uniquely enable sequencing of both sides of a single cleavage site in one DNA molecule using paired-end sequencing.

Optimization of this method, which we called CIRCLE-seq, and characterization of its technical reproducibility **(Fig. 1D)**, was performed as follows. To achieve restriction-enzyme independent circularization of genomic DNA, we tested a strategy based on ligation of a uracil-containing stem loop adapter to a end-repaired, A-tailed PCR amplicon. We enzymatically selected for covalently-closed DNA molecules that had stem-loop adapters ligated to both sides with a mixture of Lambda exonuclease and *E. coli* exonuclease I. 4 bp overhangs were released using a mixture of USER enzyme and T4 PNK, ligation was performed with T4 DNA ligase under conditions favoring intramolecular ligation, and successful circularization was measured by capillary electrophoresis. The conditions resulting in highest circularization efficiency (400 U T4 DNA ligase, 5 ng/ul DNA concentration) were

used for subsequent experiments. To characterize the technical reproducibility of CIRCLE-seq, we performed independent library preparations from the same source of U2OS genomic DNA. We observed strong CIRCLE-seq read count correlations in independent technical replicates (**Fig. 1D**).

Example 2. CIRCLE-seq enables highly sensitive *in vitro* detection of CRISPR-Cas9 genome-wide off-target cleavage sites

To test the efficacy and sensitivity of CIRCLE-seq, we used it to identify off-target cleavage sites of Cas9 directed by the single published gRNA that has been profiled with the most recent and accurate version of Digenome-seq to date[35]. CIRCLE-seq evaluation of SpCas9 with this gRNA (targeted to the human *HBB* gene) on human K562 cell genomic DNA identified not only 26 of the 29 off-target sites previously identified by GUIDE-seq but also 156 new off-target sites (**Fig. 2A**). For the three off-target sites found by Digenome-seq but not by CIRCLE-seq, we observed supporting reads in the CIRCLE-seq data, demonstrating that these sites were simply undersampled in these particular experiments. Of the 156 new off-target cleavage sites detected only by CIRCLE-seq, we found that 29 of these also showed evidence of cleavage in the original Digenome-seq data[34] (**Fig. 2B**); the inability of Digenome-seq to call these sites is most likely due to stringent informatics scoring criteria required to contend with the abundant genome-wide background reads generated by this method. By contrast, we found that such background reads were rare with CIRCLE-seq (**Fig. 2C**). Indeed, we estimate the enrichment factor of CIRCLE-seq for nuclease-cleaved sequence reads to random background reads is ~180,000-fold better than that of Digenome-seq based on examinination of an on-target site with the two methods and adjusting for sequencing depth (**Fig. 2C**). The start mapping positions of bidirectional CIRCLE-seq reads are consistent with the expected cleavage site of SpCas9 (3 bp before the PAM sequence) (**Fig. 2D**), demonstrating the ability of this method to precisely map cleavage positions with nucleotide-level precision. Taken together, these results demonstrate that CIRCLE-seq possesses higher signal-to-noise relative to Digenome-seq, which most likely accounts for its greater sensitivity for identifying genome-wide off-target sites.

Example 3. Direct comparisons of CIRCLE-seq with cell-based off-target determination methods

We next compared the performance of CIRCLE-seq with GUIDE-seq, one of the most sensitive cell-based approaches currently available for genome-wide off-target mutation identification[30]. In an initial comparison, we used CIRCLE-seq to assess SpCas9 with six different gRNAs targeted to standard non-repeitive sequences and that had been previously characterized by GUIDE-seq across two different human cell lines. CIRCLE-seq identified variable numbers of off-target cleavage sites for these six different gRNAs, ranging in number from as few as 21 to as many as 124 (**Fig. 3A;** Exemplary data for one replicate of an EMX1 targeting experiment is shown in Table 2, below; EMX1 target site sequence: GAGTCCGAGCAGAAGAAGAANGG (SEQ ID NO:2)) and distributed throughout the human genome (**Fig. 3B**). Importantly, for all six gRNAs, CIRCLE-seq identified many more off-target sites than previously found by GUIDE-seq, including the gRNA targeted to RNF2 for which we had previously been unable to identify off-target sites using GUIDE-seq. For four of the six gRNAs, CIRCLE-seq detected all of the off-target sites identified by GUIDE-seq (**Fig. 3C**) and for the other two gRNAs, it detected all but one off-target for each (**Fig. 3C**). Closer examination of the CIRCLE-seq data for these experiments actually revealed evidence of some supporting reads for these two off-target sites but not of a sufficient number required to meet our statistical threshold for detection in these experiments. In addition, as might be expected, these two undetected sites had been at the edge of detection in our GUIDE-seq experiments. Taken together, these findings again suggest that these two off-target sites would be detected if we modestly increased CIRCLE-seq sequencing depth.

To provide a more challenging test of CIRCLE-seq, we also profiled SpCas9 with four additional gRNAs that are targeted to repetitive sequences and that had been previously characterized by GUIDE-seq. Due to the repetitive nature of their targets, these four gRNAs have a relatively larger number of closely matched sites in the human genome (**Table 1**) and, not surprisingly, have been shown by GUIDE-seq to induce a large number of off-target effects in human cells[30]. As expected, CIRCLE-seq also identified a much larger number of off-target sites, ranging in number from 496 to 2503 for each of the four gRNAs (**Fig. 3A** and **Table 2**) and distributed throughout the human genome (**Fig. 3B**). Included among these were 353 of the 364 off-target sites previously identified by GUIDE-seq experiments (**Fig. 3C**). For 9 of

the 11 sites found by GUIDE-seq but not identified by CIRCLE-seq, evidence of supporting reads could be found in the CIRCLE-seq data but not of a sufficiently high number for statistical cutoff, once again suggesting that greater sequencing read depth would enable detection of these sites.

We next used CIRCLE-seq to profile SpCas9 and two gRNAs that had previously been characterized by the cell-based HTGTS method. These experiments revealed that, for both gRNAs (targeted to sites in the *EMX1* and *VEGFA* genes), CIRCLE-seq found 50 of the 53 off-target sites (94%) previously identified by HTGTS (**Fig. 3E**). Among the three HTGTS sites not detected by CIRCLE-seq, two were found when additional experimental replicates were performed and the third had a low HTGTS score, suggesting that these three sites would be detected with greater CIRCLE-seq sequencing depth. Importantly, CIRCLE-seq also found a much greater number of off-target sites than had been previously identified by HTGTS (**Fig. 3D**).

Table 1.

		Numbers of in silico off-target sites predicted in the human genome								
Target Site Sequence	**Target site**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
GAGTCCGAGCAGAAGAAGAANGG (SEQ ID NO:2)	EMX1	1	1	2	27	421	4313	34761	218047	1156729
GGAATCCCTTCTGCAGCACCNGG (SEQ ID NO:3)	FANCF	1	1	3	33	449	3155	21793	135144	724696
GTCATCTTAGTCATTACCTGNGG (SEQ ID NO:4)	RNF2	1	1	1	11	204	2029	18023	138077	830825
GGGAAAGACCCAGCATCCGTNGG (SEQ ID NO:5)	Site_1	1	1	2	14	132	1499	13410	99120	627262
GAACACAAAGCATAGACTGCNGG (SEQ ID NO:6)	Site_2	1	1	2	16	239	3075	27129	180822	1026201
GGCCCAGACTGAGCACGTGANGG (SEQ ID NO:7)	Site_3	1	1	2	16	156	1831	15689	112679	645364
GGCACTGCGGCTGGAGGTGGNGG (SEQ ID NO:8)	Site_4	1	1	10	125	1231	9452	56139	297118	1471381
GGGTGGGGGGAGTTTGCTCCNGG (SEQ ID NO:9)	VEGFA_site_1	1	2	6	51	442	3870	28723	178630	929570
GACCCCCTCCACCCCGCCTCNGG (SEQ ID NO:10)	VEGFA_site_2	1	1	10	58	726	7636	51673	305299	1469770
GGTGAGTGAGTGTGTGCGTGNGG (SEQ ID NO:11)	VEGFA_site_3	1	2	37	1077	24857	530932	921004	1538579	2944099

Table 2

Chromosome:Start-End	Read	Strand	Off-target Sequence	SEQ ID NO:	Distance	Length
2:73160981-73161004	1004	+	GAGTCCGAGCAGAAGAAGAAGGG	12.	0	23
8:120587494-120587517	746	-	AAGGCCAAGCAGAAGAGTAATGG	13.	5	23
8:128801241-128801264	478	+	GAGTCCTAGCAGGAGAAGAAGAG	14.	3	23
2:219845055-219845078	466	+	GAGGCCGAGCAGAAGAAAGACGG	15.	3	23
3:5031597-5031620	226	+	GAATCCAAGCAGGAGAAGAAGGA	16.	4	23
3:95690179-95690202	182	-	TCATCCAAGCAGAAGAAGAAGAG	17.	5	23
2:218378101-218378124	180	-	GAGTCTAAGCAGGAGAATAAAGG	18.	4	23
1:23720611-23720634	160	-	AAGTCCGAGGAGAGGAAGAAAGG	19.	3	23
1:234492858-234492881	158	-	GAAGTAGAGCAGAAGAAGAAGCG	20.	5	23
1:33606473-33606496	152	-	GAGCCTGAGCAGAAGGAGAAGGG	21.	3	23
11:43747931-43747954	146	+	AAGCCCGAGCAAAGGAAGAAAGG	22.	4	23
12:106646073-106646096	140	+	AAGTCCATGCAGAAGAGGAAGGG	23.	4	23
5:45359060-45359083	136	-	GAGTTAGAGCAGAAGAAGAAAGG	24.	2	23
1:21580957-21580980	134	-	TCTTCCAAGCAGAGGAAGAAAGG	25.	5	23
10:128080178-128080201	132	+	GAGTACAAGCAGATGAAAAACGG	26.	4	23
14:43156800-43156823	130	-	GAGGCCAAGCAGAAAAAAAATGG	27.	4	23
2:172374197-172374220	128	-	GAAGTAGAGCAGAAGAAGAAGCG	28.	5	23
20:6653992-6654015	126	-	AAGTCCAGACAGAAGAAGAAGGA	29.	5	23
16:78848843-78848866	116	-	AAATCCAACCAGAAGAAGAAAGG	30.	4	23
15:44109746-44109769	114	+	GAGTCTAAGCAGAAGAAGAAGAG	31.	3	23
7:141972555-141972578	114	-	AAGTCCGGGCAAAAGAGGAAAGG	32.	4	23
7:31901071-31901094	108	-	GAGGCCAAGCAGAAAGAAAAAGG	33.	5	23
1:27913373-27913396	102	+	AGGTCAGAGCAGAAGAAAAGAGG	34.	5	23

Table 2

Chromosome:Start-End	Read	Strand	Off-target Sequence	SEQ ID NO:	Distance	Length
14:48932102-48932125	100	+	GAGTCCCAGCAAAAGAAGAAAAG	35.	3	23
4:48639390-48639413	92	+	CACTCCAAGTAGAAGAAGAAAAG	36.	5	23
10:91416144-91416167	90	+	ATGTCCAAGCAGAAGAAGTCTGG	37.	5	23
2:54284994-54285017	90	+	AAGGCAGAGCAGAGGAAGAGAGG	38.	5	23
15:100292461-100292484	82	+	AAGTCCCGGCAGAGGAAGAAGGG	39.	4	23
1:151027591-151027614	80	-	TTCTCCAAGCAGAAGAAGAAGAG	40.	5	23
19:1438808-1438831	80	+	GAAGTAGAGCAGAAGAAGAAGCG	41.	5	23
19:24250496-24250519	76	-	GAGTCCAAGCAGTAGAGGAAGGG	42.	3	23
12:73504668-73504691	74	-	GAGTTAGAGCAGAAAAAAAATGG	43.	4	23
11:62365266-62365289	72	-	GAATCCAAGCAGAAGAAGAGAAG	44.	4	23
14:75723901-75723924	72	-	AGTTCCAAGCAGAGGAAGAAGGG	45.	5	23
10:58848711-58848734	70	+	GAGCACGAGCAAGAGAAGAAGGG	46.	4	23
7:83262530-83262552	66	-	AAGTCAGAG-AGAAGAAGAAGAG	47.	4	22
11:30301802-30301825	58	-	CAGTCTGAGTAGAAGAAAAAGGG	48.	4	23
5:9227145-9227168	58	+	AAGTCTGAGCACAAGAAGAATGG	49.	3	23
5:146833183-146833206	58	-	GAGCCGGAGCAGAAGAAGGAGGG	50.	3	23
6:9118792-9118815	56	-	ACGTCTGAGCAGAAGAAGAATGG	51.	3	23
7:100895235-100895258	56	-	CGCTCCGAGCAGAAGAAAAGTGG	52.	5	23
8:26500632-26500655	54	+	CCATCCGAGCAGGAGATTAATGG	53.	6	23
7:111475442-111475465	54	-	GAGCCCAAGCACAAAAAGAATGG	54.	4	23
4:87256685-87256708	52	-	GAGTAAGAGAAGAAGAAGAAGGG	55.	3	23
9:34735034-34735057	52	+	AAGTCCTAGCAGAAGGAAAGGGG	56.	5	23
2:14945887-14945910	50	-	AAGTCCAGGCAGGAGAAAAATGG	57.	5	23
19:56089509-56089532	48	-	AAGGCCGAGCAGGAGGAAGAAGG	58.	6	23

Table 2

Chromosome:Start-End	Read	Strand	Off-target Sequence	SEQ ID NO:	Distance	Length
4:44622959-44622982	46	+	AAGTCTGAGAAGAAGAAAGA	59.	4	23
4:60950632-60950655	46	+	TATTCCAAGCAAAAGAAAAAGGG	60.	5	23
2:163702353-163702376	44	+	AAGCCCAAGCAGAAGAAAAATGA	61.	5	23
17:78609059-78609082	40	-	GAGCCCGTGCAGAGGAAGAAGGA	62.	4	23
3:45605380-45605403	36	-	GAGTCCACACAGAAGAAGAAAGA	63.	4	23
3:149083982-149084005	36	+	CTGTCCAAGCACAAGAACAATGG	64.	5	23
1:221522607-221522630	34	+	GAGTTTGAGTAGAAGAAGAAGAG	65.	4	23
12:114102185-114102208	34	+	AGGTCTGAGCAGAAGAAAGAAGG	66.	5	23
5:120294729-120294752	34	-	ATGTCCAAGCACAAGAGGAATGG	67.	5	23
8:105164108-105164131	34	+	GAGCCCAAGAAGAAGAAGAAGGA	68.	4	23
15:56583426-56583449	30	+	AAGTCTGAGTAGGAGAAAAAGGG	69.	5	23
19:51616603-51616626	30	-	AGGTCTGAGCAGAAGAGGAAGAG	70.	5	23
4:174898812-174898835	30	-	TGATCCAAGCAGGAGAAAAAATGG	71.	6	23
3:123771739-123771762	30	+	CATTCCTAGCAGAGGAAGAAAGG	72.	4	23
10:5401770-5401793	28	+	TAATCCAATCAGAAGAAGAAGGG	73.	4	23
6:961042-961065	28	+	AAATCCAACCCAGAAGAAGAGGGG	74.	5	23
16:5501173-5501196	28	-	CAATCAGAGCAGAGGAAGAAGAG	75.	5	23
2:200422147-200422170	26	-	GAAGCCAAGCAGAAGAAAAAACAG	76.	5	23
2:224569568-224569591	26	+	GAGGCTGAGCAGAAGAGGAAGGA	77.	4	23
5:16467414-16467437	26	-	TGTTCCAAGCAGAAGAGTAATGG	78.	6	23
8:10048559-10048582	26	-	TAGTCTAAGCAGCAGAAGAATGG	79.	4	23
21:24667736-24667759	24	-	CCCTCCAAGCAGAAGAAGATGAG	80.	6	23
3:13455177-13455200	24	+	AGGCCCGAGCAGGAGAAAATAGG	81.	6	23
1:201617280-201617303	22	-	AGCTCCGAGCAGAGGAAGGAGGG	82.	5	23

Table 2

Chromosome:Start-End	Read	Strand	Off-target Sequence	SEQ ID NO:	Distance	Length
1:231750724-231750747	22	+	GAGTCAGAGCAAAAGAAGTAGTG	83.	4	23
2:203707473-203707496	22	-	GAGTTTAAGCAGAAGAAGAGAGG	84.	4	23
8:97023219-97023242	22	+	TCTTCCAAGCAAAAGAAGAAAGA	85.	6	23
9:72006952-72006975	22	-	GAGGCCCAGCAGAGGAAGAAGAG	86.	4	23
9:127309498-127309521	22	-	AAGCCCAAGCAAATGAAGAATGG	87.	5	23
16:73177721-73177744	22	+	TCTTCCGAGCTGAAGAAGAAAAG	88.	5	23
2:65782509-65782532	20	+	GACTCCGAGCAGCAGAAGGATGG	89.	3	23
16:8265310-8265333	20	+	GAGACCAAACAGAGGAAGAAGGG	90.	4	23
7:3812774-3812797	20	-	GAGTCCTAGAAAAAGAAGAGAGG	91.	4	23
7:70109949-70109972	20	+	GAATCAGAGCAAAAGGAGAAAGG	92.	4	23
1:209931582-209931605	18	+	TTATCCGAGAAGAAGAAGTAAGG	93.	5	23
22:34302975-34302998	18	+	AATTCCAAGCAGAAGAAAAAGGA	94.	5	23
8:102244534-102244557	18	+	AGTTCCAAGCAGAAGAAGCATGG	95.	5	23
3:57591866-57591889	18	+	AAGTCCAAGCACAAGAAACATGG	96.	5	23
17:54421036-54421059	16	-	GAGTCCCAGGAGAAGAAGAGAGG	97.	3	23
2:66729755-66729778	16	+	AGTTCAGAGCAGGAGAAGAATGG	98.	5	23
4:21141327-21141350	16	-	AAGCCCGAGCAGAAGAAGTTGAG	99.	5	23
6:99699155-99699178	16	-	GAGTTAGAGCAGAGGAAGAGAGG	100.	4	23
9:135663386-135663409	16	+	CAGTCCAAACAGAAGAGGAATGG	101.	4	23
X:53467704-53467727	16	-	GAGTCCGGGAAGGAGAAGAAAGG	102.	3	23
21:32643345-32643368	16	-	AAGGCAAAGCAAAAGAAGAGGGG	103.	6	23
1:113741452-113741475	14	+	GAGGTAGAGCAGAAGAAGAAGCG	104.	4	23
10:63704768-63704791	14	-	AAGTCCCAGCAACAGAAGAAAGG	105.	4	23
12:119985924-119985947	14	-	GACTCCTAGCAAAAGAAGAATGG	106.	3	23

Table 2

Chromosome:Start-End	Read	Strand	Off-target Sequence	SEQ ID NO:	Distance	Length
19:44223469-44223492	14	+	GAGGCCTTGCAGAAGAAGAAGGC	107.	4	23
20:23516684-23516707	14	-	GAATCCCAGCAGGAGAGGAATGG	108.	4	23
5:53966504-53966527	14	-	CAATCCGGGCAGAAGAAGGAGAG	109.	5	23
8:74634205-74634228	14	-	AAGTCCAAAAAGAAGAAAAAAGG	110.	5	23
18:15077483-15077506	14	+	TAGGCTGAGCAGAAGAAAAAGGA	111.	5	23
14:38654648-38654671	12	+	AAGTCTGAGAAGAAGAAGACATG	112.	5	23
14:50538462-50538485	12	-	TAGTCCTAGCAAAAGCAGAAGGG	113.	4	23
4:131662215-131662237	12	-	GAATCCAAG-AGAAGAAGAATGG	114.	3	22
6:40361640-40361663	12	+	GAGTCTAAGCAGAAGAGGACTGG	115.	4	23
18:71029915-71029938	12	-	GAGTCCCAGCAGGAGAAGAAAGA	116.	3	23
3:63468110-63468133	12	+	AAGTTGGAGCAGGAGAAGAAGGG	117.	4	23
1:35385584-35385607	10	+	GAAGTGGAGCAGGAGAAGAAGGG	118.	5	23
11:68772640-68772663	10	-	GAGTCCATACAGGAGAAGAAAGA	119.	5	23
12:32650839-32650861	10	-	GAGTC-GAGAAGAAGAAAAAAGG	120.	3	22
12:70327491-70327514	10	-	GAATCCCAGCAGGAGAAGACAGG	121.	4	23
13:27769640-27769663	10	+	GAGTAGGAGCAGGAGAAGAAGGA	122.	4	23
14:20246485-20246507	10	+	GAGTA-GAGCAGAGGAGGAAGGG	123.	4	22
14:94517597-94517620	10	-	CTCTCCAAGCAGAAGAAGAAGAA	124.	6	23
17:74877554-74877577	10	+	GAGGCCGGGCAGGAGAAGGAGGG	125.	4	23
19:16132593-16132616	10	+	GCATCCAAGCAGGAGGAGAAGGG	126.	5	23
19:46265337-46265360	10	+	AAGCCCAAGGAGAAGAAGAAAGG	127.	4	23
2:51907191-51907214	10	+	AAGTCAAAGCAGGAGAAGAAAGA	128.	5	23
20:22037218-22037241	10	-	GAGAGAGAGCAAAAAAAAGAAGGG	129.	5	23
20:48873417-48873440	10	-	AAGCCCGGGCAGAAGAAGCACAG	130.	5	23

Table 2

Chromosome:Start-End	Read	Strand	Off-target Sequence	SEQ ID NO:	Distance	Length
4:25060740-25060763	10	-	GTGTCAGAGCAGAAAAAGAGTGG	131.	4	23
4:54651562-54651585	10	-	CATTCCAAGCAGCAGAAGAAGAG	132.	5	23
5:82309812-82309835	10	+	AAGTCCAAGCATAAGAAAACAGG	133.	5	23
8:37644281-37644304	10	+	GAGAGAGAGCAGGAGAGGAAAGG	134.	5	23
8:109199391-109199414	10	-	GAGTCAGAGCAGAAGAAAGAGGA	135.	4	23
7:19457141-19457164	10	-	ATCTCCAAGCAGAAGAAAAATGG	136.	5	23

Example 4. Mutation of off-target sites identified by CIRCLE-seq in human cells

An important question raised by our experiments is whether the novel off-target cleavage sites identified *in vitro* by CIRCLE-seq (and not by GUIDE-seq or HTGTS) are actually mutated in cells by Cas9/gRNA complexes. Given that many of the off-target sites detected by both CIRCLE-seq and GUIDE-seq have high numbers of CIRCLE-seq sequencing read counts (**Fig. 4A**), this strongly suggests GUIDE-seq is mostly detecting sites that are efficiently cleaved *in vitro*. By contrast, the off-target sites found only by CIRCLE-seq have lower CIRCLE-seq read counts (**Fig. 4A**), suggesting that these might be missed by GUIDE-seq because they are cleaved at lower frequencies. If this were correct, we would expect it to be difficult to validate these sites in cells using standard targeted amplicon sequencing because the error rate of next-generation sequencing places a floor for indel mutation detection of approximately 0.1%.

To determine whether the novel off-target sites revealed only by CIRCLE-seq (but not in our original GUIDE-seq experiments) might be cleaved in human cells, we reasoned that we could perform high depth targeted amplicon sequencing using genomic DNA obtained from cell-based GUIDE-seq experiments and look for tag integration as evidence for off-target Cas9 cleavage (**Fig. 4B**). This strategy sidesteps the problem of the indel error rate associated with deep sequencing because tag integration occurs with a negligible background frequency. Using this targeted tag integration sequencing approach, we examined a total of 98 off-target sites found by CIRCLE-seq (but not by GUIDE-seq) with SpCas9 for the *EMX1* and *VEGFA* site 1 gRNAs. We chose sites that had a range of CIRCLE-seq read counts and/or number of mismatches relative to the on-target site. As positive controls for the tag integration assay, we also selected a smaller set of off-target sites that exhibited variable CIRCLE-seq read counts and that had been found by both CIRCLE-seq and GUIDE-seq. Targeted amplicon sequencing revealed detection of the dsODN tag at all of the control off-target sites, with frequencies that correlated well with GUIDE-seq read counts (**Figs. 4C** and **4D**). Notably, we also detected dsODN tag integration at 24 of the 98 novel off-target sites identified only by CIRCLE-seq (**Figs. 4C-E**), with frequencies in the low range (0.003 – 0.2%) as anticipated. The locations of all 24 of

these tag integrations map to the expected position in the protospacer complementarity region, 3 bps away from the PAM sequence, consistent with these sites representing *bona fide* off-target cleavage sites **(Fig. 4F)**.

Example 5. Reference genome-independent off-target site discovery by CIRCLE-seq

Because each pair of CIRCLE-seq reads from a single DNA molecule yields sequences from both sides of a CRISPR-Cas9 nuclease cleavage site, we reasoned that the method might be used to identify off-target sites even without a reference genome sequence. To this end, we developed a mapping-independent off-target site discovery algorithm that merges paired-end reads in an end-to-end orientation and directly searches for off-target cleavage sites that resemble the on-target site (see **Materials and Methods**). Using this algorithm, we identified on average ~99.5% of CIRCLE-seq sites with more than 10 CIRCLE-seq reads detected by our standard reference-based mapping algorithm **(Fig. 4G)**. This result demonstrates that CIRCLE-seq can be used in a reference-independent fashion to identify off-target cleavage sites for organisms whose genome sequences are less well-characterized and/or show high genetic variability (e.g., non-inbred species in the wild).

Example 6. Association of CIRCLE-seq off-target sites with SNPs

With its higher throughput and ease of reproducibility, an *in vitro* method such as CIRCLE-seq provides the opportunity to define patient-specific off-target profiles for any given Cas9/gRNA nuclease. A previous study identified a single example of a SNP influencing off-target cleavage[36]. To more broadly test whether genetic differences can influence nuclease-induced off-target cleavage, we performed additional CIRCLE-seq experiments on human K562 genomic DNA with six gRNAs we had already assessed on human HEK293 and U2OS genomic DNAs (three gRNAs on HEK293s and three on U2OS). Although many off-target sites for these gRNAs showed correlated CIRCLE-seq read counts on DNA from both cell types tested, we also observed 55 sites that were preferentially cleaved only in one cell type or the other **(Fig. 5A)**. Examination revealed that eight off-target cleavage sites harbored non-reference single-nucleotide polymorphisms (SNPs) that might account for some of these observed cell-type specific differences in cleavage efficiencies **(Fig. 5B)**. Interestingly, these SNPs were observed in regions of protospacer complementarity as

well as within the PAM and were predicted to either increase or decrease cleavage of the site relative to the reference genome sequence **(Fig. 5B)**.

Having identified additional examples where SNPs appear to influence cleavage efficiencies at off-target sites, we next sought to estimate how frequently SNPs might be expected to impact off-target cleavage efficiency. To do this, we examined the genotypes of 2504 individuals from the 1000 Genomes Project[37] at all 1247 off-target sites we detected for the six gRNAs (targeted to standard non-repetitive sequences) we had assessed by CIRCLE-seq. We found, on average, non-reference genetic variation in ~2.5% of these off-target sites **(Fig. 5C)**. At a population level, we found that superpopulations contained genetic variation in an average of ~20% of these off-target sites **(Fig. 5C)**. In addition, 50% of these off-target sites contained non-reference genetic variation for at least one individual sequenced in the 1000 Genomes Project **(Fig. 5C)**. These frequencies are consistent with the expectation that, given existence of ~100 million validated human SNPs in the most recent version of dbSNP[38], one might expect to find a SNP in ~69% of SpCas9 off-target sites in the human genome. As expected, the range of mismatches observed at the off-target sites we examined is increased when considering diverse individual genotypes from the 1000 genomes project **(Fig. 5D)**. Interestingly, for approximately 9% of the off-target site haplotypes, the numbers of mismatches relative to the reference genome is decreased which would predict a potential increase in off-target cleavage risk at these sites **(Fig. 5E)**. Taken together, these results highlight the importance of individual genetic variation for off-target analysis and highlight how CIRCLE-seq might be used to produce personalized genome-wide off-target profiles.

Example 7. Exemplary CIRCLE-Seq protocols

Described herein are exemplary protocols for an *in vitro* assay for finding cleavage sites of CRISPR/Cas9 nuclease from complex genomic DNA mixtures.

Materials

The following materials were used in Protocol 1.

Materials	Vendor	Model Number
HTP Library Preparation Kit	Kapa Biosystems	KK8235
Hifi HotStart ReadyMix, 100 x 25 µL reactions	Kapa Biosystems	KK2602
Lambda exonuclease (5 U/ul)	NEB	M0262L

E. Coli Exonuclease I (20 U/ul)	NEB	M0293S
USER enzyme (1000 U/ul)	NEB	M5505L
T4 PNK (10000 U/ul)	NEB	M0201S
Cas9 enzyme (1000 nM)	NEB	M0386L
Ampure XP 60 ml	Agencourt	A63881

Protocol 1

1. End Repair

Component	**1 rxn (ul)**
Water	8
10X Kapa End Repair Buffer	7
Kapa End Repair Enzyme Mix	5
Total master mix volume	20
Input DNA (0.1 - 5 ug) sheared to an average of about 300 bp	50
Total reaction volume	70

Incubate 30 minutes at 20C.
1.7X SPRI cleanup using 120 ul Ampure XP beads.

2. A-tailing

Component	**1 rxn (ul)**
10X Kapa A-Tailing Buffer	5
Kapa A-Tailing Enzyme	3
Total master mix volume	8
TE (0.1 mM EDTA)	42
End repaired DNA with beads	0
Total reaction volume	50

Incubate for 30 min at 30C.
Cleanup by adding 90 ul of SPRI solution.

3. 5' Adapter Ligation

Component	**1 rxn (ul)**
5X Kapa Ligation Buffer	10
Kapa T4 DNA Ligase	5
Total master mix volume	15
A-tailed DNA with beads	0
TE (0.1 mM EDTA)	30

Stem-Loop Adapter (SEQ ID NO:1) (40 uM)	5
Total reaction volume	50

Incubate for 1 hour at 20C.
Cleanup by adding 50 ul of SPRI solution.

Proceed with 1 ug of 5' Adapter-ligated DNA into next step.

4. Lambda Exonuclease/E. Coli Exonuclease treatment

Component	**1 rxn (ul)**
10X ExoI buffer	5
Lambda exonuclease (5U/ul)	4
E. Coli Exonuclease I (20U/ul)	1
Total master mix volume	10
Adapter-ligated DNA with beads	0
TE (0.1 mM EDTA)	40
Total reaction volume	50

Incubate for 1 hour at 37C, 75C for 10 min.
Cleanup by adding 90 ul of 1X Ampure XP beads.

5. USER/T4 PNK treatment

Component	**1 rxn (ul)**
10X T4 DNA ligase buffer	5
USER enzyme	3
T4 PNK	2
Total master mix volume	10
Adapter-ligated DNA with beads	0
TE (0.1 mM EDTA)	40
Total reaction volume	50

Incubate for 1 hour at 37C.
Cleanup by adding 90 ul of SPRI solution.

6. Intramolecular Circularization

Component	**1 rxn (ul)**
10X T4 DNA ligase buffer	10
T4 DNA ligase (400U/ul)	2
H2O	8
Total master mix volume	20

Adapter-ligated, USER/T4 PNK-treated DNA (500 ng)	80
Total reaction volume	100

Incubate overnight (16 hours) at 16C.
Cleanup by adding 100 ul of Ampure XP beads

7. PlasmidSafe Exonuclease treatment

Component	**1 rxn (ul)**
10X PlasmidSafe Buffer	5
25 mM ATP	2
PlasmidSafe enzyme	5
Total master mix volume	12
Adapter-ligated DNA with beads	0
TE (0.1 mM EDTA)	38
Total reaction volume	50

Incubate for 1 hour at 37C, 70C for 30 min.
Cleanup by adding 50 ul of 1X Ampure XP beads.

8. Cleavage by Cas9

Component	**1 rxn (ul)**
Water	63
10X Cas9 buffer	10
Cas9 (1 uM -> 900 nM final)	9
sgRNA (300 nM, ~100 ng/ul)	3
Total reaction volume	85

Incubate at room temperature for 10 minutes.
DNA (~400 bp, 250 ng) 15
Incubate for 1 hour at 37C.
Purify with 1X SPRI bead cleanup (100 ul).

9. A-tailing

Component	**1 rxn (ul)**
10X Kapa A-Tailing Buffer	5
Kapa A-Tailing Enzyme	3
Total master mix volume	8
TE (0.1 mM EDTA)	42
End repaired DNA with beads	0
Total reaction volume	50

Incubate for 30 min at 30C.
Cleanup by adding 90 ul of SPRI solution.

10. 3' Adapter Ligation

Component	**1 rxn (ul)**
5X Kapa Ligation Buffer	10
Kapa T4 DNA Ligase	5
Total master mix volume	15
A-tailed DNA with beads	0
TE (0.1 mM EDTA)	30
NEBNext Adapter (15 uM)	5
Total reaction volume	50

Incubate for 30 minutes at 20C.
Cleanup by adding 50 ul of SPRI solution.

11. USER enzyme treatment

Add 3 ul of USER enzyme and treat for 60 minutes at 37C.
(Treatment is in TE 10 mM Tris, 0.1 mM EDTA.)

Purify with 1X SPRI solution (50 ul).

9. PCR amplification

Reagent	**Vendor**
Gentra Puregene Tissue Kit	Qiagen
Qubit dsDNA BR Assay Kit	Thermo Fisher
Agencourt AMPure XP magnetic beads	Beckman Coulter
High throughput, "with bead", PCR-free Library Preparation Kit	KAPA Biosystems
Enzymes and buffers - Lamda exonuclease - Exonuclease I (*E.coli*) - USER enzyme - T4 polynucleotide kinase - T4 DNA Ligase - Cas9 nuclease, *S. pyogenes*	New England Biolabs
Plasmid-Safe™ ATP-Dependent DNase	Epicentre
MEGAshortscript™ Kit	Thermo Fisher
NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 1)	New England Biolabs

KAPA HiFi HotStart ReadyMix	KAPA Biosystems
ddPCR™ Library Quantification Kit for Illumina TruSeq	Bio-Rad
KAPA Library Quantification Kit for NGS (Universal)	KAPA Biosystems

CIRCLE-seq Hairpin Adapter

oSQT1288 /5Phos/CGGTGGACCGATGATC /ideoxyU/ ATCGGTCCACCG*T

Annealing Program: 95°C for 5 min, -1°C/min for 70 cycles, hold at 4°C.

Input Quantification and Shearing

1. Genomic DNA is sheared to an average length of 300 bp according to the standard operating protocol for the Covaris S2.
2. Sheared DNA is cleaned up with 1.8X Ampure XP SPRI beads according to manufacturer protocol, and eluted in 35 µl of 1X TE buffer.

End-repair

1. For each end-repair reaction:

Component	Volume
Nuclease-free H_2O	8 µl
KAPA End Repair Buffer (10X)	7 µl
KAPA End Repair Enzyme Mix	5 µl
Total Master Mix	**20 µl**
Sheared genomic DNA (5 µg) (from step 2)	50 µl
Total	**70 µl**

End Repair Program: 20°C for 30 min, hold at 4°C.

2. 1.7X SPRI cleanup (120 µl of Agencourt Ampure XP beads), elute in 42 µl of 1X TE buffer.

A-tailing

1. For each A-tailing reaction:

Component	Volume
KAPA A-tailing Buffer (10X)	5 µl
KAPA A-tailing Enzyme	3 µl
Total Master Mix	**8 µl**
End Repaired DNA with beads (from step 4)	42 µl
Total	**50 µl**

A-tailing Program: 30°C for 30 min, hold at 4°C.

2. 1.8X SPRI cleanup (90 µl of PEG/NaCl SPRI Solution), elute in 30 µl of 1X TE buffer.

Adapter Ligation

1. For each ligation reaction to annealed adapter oSQT1288:

Component	Volume
KAPA Ligation Buffer (5X)	10 µl
KAPA T4 DNA Ligase	5 µl
Annealed Hairpin Adapter oSQT1288 (40 µM)	5 µl
Total Master Mix	**20 µl**
A-tailed DNA with beads (from step 6)	30 µl
Total	**50 µl**

Ligation Program: 20°C for 1 hr, hold at 4°C.

2. 1X SPRI cleanup (50 µl of PEG/NaCl SPRI Solution), elute in 30 µl of 1X TE buffer.

Enzymatic Treatments

Lambda Exonuclease/Exonuclease I (*E.coli*) Treatment

1.

Component	Volume
Exonuclease I Reaction Buffer (10X)	5 µl
Lambda Exonuclease (5 U/µl)	4 µl
Exonuclease I (*E.coli*) (20 U/µl)	1 µl
Total Master Mix	**10 µl**
Adapter ligated DNA (1 µg) (from step 8)	40 µl
Total	**50 µl**

Incubation Program: 37°C for 1 hr, 75°C for 10 min, hold at 4°C.

2. 1.8X SPRI cleanup (90 µl of Agencourt Ampure XP beads), elute in 40 µl of 1X TE buffer.

USER/T4 PNK Treatment

1.

Component	Volume
T4 DNA Ligase Buffer (10X)	5 µl
USER Enzyme (1 U/µl)	3 µl
T4 Polynucleotide Kinase (10 U/µl)	2 µl
Total Master Mix	**10 µl**
Lambda Exonuclease/Exonuclease I treated DNA with beads (from step 10)	40 µl
Total	**50 µl**

Incubation Program: 37°C for 1 hr, hold at 4°C.

2. 1.8X SPRI cleanup (90 µl of PEG/NaCl SPRI Solution), elute in 35 µl of 1X TE buffer.

Intramolecular Circularization

1.

Component	Volume
Nuclease-free H_2O	8 µl
T4 DNA Ligase Buffer (10X)	10 µl
T4 DNA Ligase (400 U/µl)	2 µl
Total Master Mix	**20 µl**
USER/T4 PNK treated DNA (500 ng) (from step 12)	80 µl
Total	**100 µl**

Circularization Program: 16°C for 16 hrs.

2. 1X SPRI cleanup (100 µl of Agencourt Ampure XP beads), elute in 38 µl of 1X TE buffer.

Plasmid-Safe ATP-Dependent DNase Treatment

1.

Component	Volume
Plasmid-Safe Reaction Buffer (10X)	5 µl
ATP (25 mM)	2 µl
Plasmid-Safe ATP-Dependent DNase (10 U/µl)	5 µl
Total Master Mix	**12 µl**
Circularized DNA (from step 14)	38 µl
Total	**50 µl**

Incubation Program: 37°C for 1 hr, 70°C for 30 min, hold at 4°C.

2. 1X SPRI cleanup (50 µl of Agencourt Ampure XP beads), elute in 15 µl of 1X TE buffer.

In Vitro Digestion with Cas9 and gRNA

1.

Component	Volume
Cas9 Nuclease Reaction Buffer (10X)	10 µl
Cas9 Nuclease, *S. pyogenes* (1 µM)	9 µl
In Vitro Transcribed guide RNA (3000 nM)	3 µl
Total Master Mix	**22 µl**

Incubate at room temperature for 10 min.

Plasmid-Safe DNase Treated DNA (250 ng) (from step 16)	78 µl
Total	**100 µl**

Digestion Program: 37°C for 1 hr, hold at 4°C.

2. 1X SPRI cleanup (100 µl of Agencourt Ampure XP beads), elute in 42 µl of 1X TE buffer.

A-tailing

1.

Component	Volume
KAPA A-tailing Buffer (10X)	5 µl
KAPA A-tailing Enzyme	3 µl
Total Master Mix	**8 µl**
Cas9/gRNA digested DNA with beads (from step 18)	42 µl
Total	**50 µl**

A-tailing Program: 30°C for 30 min, hold at 4°C.

2. 1.8X SPRI cleanup (90 µl of PEG/NaCl SPRI Solution), elute in 30 µl of 1X TE buffer.

Adapter Ligation

1.

Component	Volume
KAPA Ligation Buffer (5X)	10 µl
KAPA T4 DNA Ligase	5 µl
NEBNext Adaptor for Illumina (15 µM) *	10 µl
Total Master Mix	**25 µl**
A-tailed DNA with beads (from step 20)	25 µl
Total	**50 µl**

* NEBNext Adaptor for Illumina (#E7601A):
5'-/5Phos/GATCGGAAGAGC ACACGTCTGAACTCCAGTC/ideoxyU/ACACTCT TT
CCTACACGACGCTCTTCCGAT C*T-3

Ligation Program: 20°C for 1 hr, hold at 4°C.

2. 1X SPRI cleanup (50 µl of PEG/NaCl SPRI Solution), elute in 47 µl of 1X TE buffer.

USER Enzyme Treatment

1. Add 3 µl of USER Enzyme (1 U/µl) to the adapter ligated DNA with beads (from step 22).
2. 0.7X SPRI cleanup (35 µl of PEG/NaCl SPRI Solution), elute in 20 µl of 1X TE buffer.

PCR

1.

Component	Volume
Nuclease-free H_2O	5 µl
KAPA HiFi HotStart ReadyMix	25 µl
Total Master Mix	**30 µl**
NEBNext i5 Primer (10 µM)	5 µl
NEBNext i7 Primer (10 µM)	5 µl
USER enzyme treated DNA (20 ng) (from step 24)	10 µl
Total	**50 µl**

PCR Program: 98°C for 45 s, 22 cycles of (98°C for 15 s, 65°C for 30 s, 72°C for 30 s), 72°C for 1 min, hold at 4°C.

2. 0.7X SPRI cleanup (35 µl of Agencourt Ampure XP beads), elute in 30 µl of 1X TE buffer.

Library Quantification

1. Quantify the library using ddPCR Library Quantification Kit for Illumina TruSeq (Bio-Rad) on QX200 Droplet Digital PCR instrument, according to the manufacturer instructions. An alternative quantification method is using KAPA Library Quantification Kit for Next-Generation Sequencing (KAPA Biosystems), according to the manufacturer instructions.

References

1. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823 (2013).

2. Hwang, W. Y. et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31, 227–229 (2013).

3. Jinek, M. et al. RNA-programmed genome editing in human cells | eLife. Elife 2, e00471 (2013).

4. Mali, P. et al. RNA-guided human genome engineering via Cas9. Science 339, 823–826 (2013).

5. Jinek, M. et al. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337, 816–821 (2012).

6. Doudna, J. A. & Charpentier, E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346, 1258096 (2014).

7. Bolukbasi, M. F., Gupta, A. & Wolfe, S. A. Creating and evaluating accurate CRISPR-Cas9 scalpels for genomic surgery. Nat Meth 13, 41–50 (2016).

8. Mali, P., Esvelt, K. M. & Church, G. M. Cas9 as a versatile tool for engineering biology. Nat Meth 10, 957–963 (2013).

9. Sander, J. D. & Joung, J. K. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32, 347–355 (2014).

10. Maeder, M. L. & Gersbach, C. A. Genome-editing Technologies for Gene and Cell Therapy. Mol Ther 24, 430–446 (2016).

11. Lin, J. & Musunuru, K. Genome Engineering Tools for Building Cellular Models of Disease. FEBS J (2016). doi:10.1111/febs.13763

12. Hsu, P. D., Lander, E. S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. Cell 157, 1262–1278 (2014).

13. Brandsma, I. & Gent, D. C. Pathway choice in DNA double strand break repair: observations of a balancing act. Genome Integr 3, 9 (2012).

14. Symington, L. S. & Gautier, J. Double-strand break end resection and repair pathway choice. Annu. Rev. Genet. 45, 247–271 (2011).

15. Kass, E. M. & Jasin, M. Collaboration and competition between DNA double-strand break repair pathways. FEBS Letters 584, 3703–3708 (2010).

16. Wyman, C. & Kanaar, R. DNA double-strand break repair: all's well that ends well. Annu. Rev. Genet. 40, 363–383 (2006).

17. Rouet, P., Smih, F. & Jasin, M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol Cell Biol 14, 8096–8106 (1994).

18. Sternberg, S. H., LaFrance, B., Kaplan, M. & Doudna, J. A. Conformational control of DNA target cleavage by CRISPR-Cas9. Nature 527, 110–113 (2015).

19. Kiani, S. et al. Cas9 gRNA engineering for genome editing, activation and repression. Nat Meth 1–6 (2015). doi:10.1038/nmeth.3580

20. Dahlman, J. E. et al. Orthogonal gene knockout and activation with a catalytically active Cas9 nuclease. Nat Biotechnol 1–4 (2015). doi:10.1038/nbt.3390

21. Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M. & Joung, J. K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat Biotechnol (2014). doi:10.1038/nbt.2808

22. Anders, C., Niewoehner, O., Duerst, A. & Jinek, M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature (2014). doi:10.1038/nature13579

23. Shah, S. A., Erdmann, S., Mojica, F. J. M. & Garrett, R. A. Protospacer recognition motifs: mixed identities and functional diversity. RNA Biol 10, 891–899 (2013).

24. Tsai, S. Q. & Joung, J. K. Defining and improving the genome- wide specificities of CRISPR–Cas9 nucleases. Nature 17, 300–312 (2016).

25. Bolukbasi, M. F., Gupta, A. & Wolfe, S. A. Creating and evaluating accurate CRISPR-Cas9 scalpels for genomic surgery. Nat Meth 13, 41–50 (2015).

26. Gori, J. L. et al. Delivery and Specificity of CRISPR-Cas9 Genome Editing Technologies for Human Gene Therapy. Hum Gene Ther 26, 443–451 (2015).

27. Cox, D. B. T., Platt, R. J. & Zhang, F. Therapeutic genome editing: prospects and challenges. Nat Med 21, 121–131 (2015).

28. Gabriel, R. et al. An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nat Biotechnol 29, 816–823 (2011).

29. Ran, F. A. et al. In vivo genome editing using Staphylococcus aureus Cas9. Nature 520, 186–191 (2015).

30. Tsai, S. Q. et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat Biotechnol 33, 187–197 (2015).

31. Frock, R. L. et al. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. Nat Biotechnol 33, 179–186 (2015).

32. Crosetto, N. et al. Nucleotide-resolution DNA double-strand break mapping by next-generation sequencing. Nat Meth 10, 361–365 (2013).

33. Pattanayak, V. et al. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nat Biotechnol 31, 839–843 (2013).

34. Kim, D. et al. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. Nat Meth (2015). doi:10.1038/nmeth.3284

35. Kim, D., Kim, S., Kim, S., Park, J. & Kim, J.-S. Genome-wide target specificities of CRISPR-Cas9 nucleases revealed by multiplex Digenome-seq. Genome Res gr.199588.115 (2016). doi:10.1101/gr.199588.115

36. Yang, L. et al. Targeted and genome-wide sequencing reveal single nucleotide variations impacting specificity of Cas9 in human stem cells. Nature Communications 5, 5507 (2014).

37. 1000 Genomes Project Consortium et al. A global reference for human genetic variation. Nature 526, 68–74 (2015).

38. Sherry, S. T. et al. dbSNP: the NCBI database of genetic variation. Nucleic Acids Res 29, 308–311 (2001).

39. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature 489, 57–74 (2012).

40. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26, 589–595 (2010).

41. Li, H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27, 2987–2993 (2011).

42. Li, H. et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of preparing a library of covalently closed circular double-stranded DNA (dsDNA) fragments, the method comprising:
providing a sample comprising dsDNA;
randomly shearing the dsDNA to a defined average length to provide a population of dsDNA fragments;
optionally preparing the fragments for end-ligation;
ligating to the ends of the fragments a stem-loop adapter comprising at least a single deoxyuridine adjacent to or within a loop sequence comprising a palindromic sequence, to prepare a population of ligated linear dsDNA fragments;
contacting the population of ligated linear dsDNA fragments with an exonuclease to degrade any remaining linear fragments with unligated ends, to produce a purified population of ligated linear dsDNA fragments,
contacting the purified population of ligated linear dsDNA fragments with enzymes that nick the ligated dsDNA fragments at the deoxyuridine and to remove a 3' terminal phosphate;
incubating the nicked linear dsDNA fragments under conditions sufficient to promote intramolecular ligation and formation of circular dsDNA molecules; and
purifying the ligated circular dsDNA fragments using an exonuclease, thereby preparing a library of covalently closed fully circular dsDNA fragments.

2. The method of claim 1, further comprising contacting the library of covalently closed fully circular dsDNA fragments with an engineered nuclease to induce site-specific cleavage;
optionally preparing the cleaved fragments for end-ligation;
ligating a sequencing adapter comprising at least a single deoxyuridine and a primer site compatible for use in PCR priming or sequencing, at the cleavage site;
contacting the library with enzymes that nick at the deoxyuridine; and
sequencing resulting fragments using primers that bind to the sequencing adapter.

3. A method of preparing a library of fragments comprising sites of engineered nuclease-induced double stranded breaks in double-stranded DNA (dsDNA) molecules, the method comprising:
providing dsDNA;

randomly shearing the dsDNA to a defined average length;
optionally end-repairing and/or A-tailing the sheared dsDNA;
ligating a stem-loop adapter, preferably comprising:
a first region, preferably of about 12 nucleotides;
a second region, preferably of about 5 nucleotides, that forms one or more loops and comprises a single deoxyuridine nucleotide adjacent to a palindromic sequence for intramolecular ligation; and
a third region that is complementary to the first region with one additional nucleotide;
contacting the library with enzymes that nick the dsDNA at the deoxyuridine and to remove a terminal 3' phosphate;
incubating the nicked dsDNA under conditions sufficient to promote intramolecular ligation and formation of a sample comprising circular dsDNA molecules;
contacting the sample with one or more exonucleases sufficient to degrade any DNA molecules that are not circular;
treating the sample with an engineered nuclease to induce site-specific cleavage;
optionally end-repairing and then A-tailing the resulting ends;
ligating a sequencing adapter comprising:
a first region of about 12 nucleotides;
a second region of about 40 nucleotides that forms one or more hairpin loops and comprises a primer site compatible for use in PCR priming and/or sequencing;
a third region of about 13 nucleotides that is complementary to the first region with one additional nucleotide; and
a single deoxyuridine nucleotide between the second and third regions,
to create a population wherein the DNA fragments that were cleaved by the nuclease have a sequencing adapter ligated to the ends;
thereby preparing a library enriched for nuclease-cleaved adapter-ligated fragments.

4. The method of claims 1 to 3, wherein randomly shearing the dsDNA comprises randomly shearing the dsDNA to an average length of about 200-500 bps, preferably about 300 bps.

5. The method of claims 1 to 3, wherein the exonuclease used to degrade any remaining linear fragments with unligated ends is a cocktail of nucleases comprising one or more of bacteriophage Lambda exonuclease, *E. coli* Exonuclease I, and an ATP-dependent exonuclease.

6. The method of claims 1 to 3, wherein preparing the fragments for end-ligation comprises one or both of end-repairing and/or A-tailing the sheared DNA.

7. The method of claims 1 to 3, wherein the enzyme that nicks DNA at a deoxyuridine comprises one or both of uracil DNA glycosylase (UDG) and/or endonuclease VIII, and the enzyme that removes the terminal 3' phosphate comprises T4 Polynucleotide Kinase.

8. The method of claims 1 to 3, wherein the DNA is genomic DNA (gDNA) or synthetic DNA.

9. The method of claims 2 or 3, wherein the engineered nuclease cleaves at on- and/or off-target sites.

10. The method of claims 2 or 3, wherein the engineered nuclease induces blunt or staggered/overhanging ends.

11. The method of claim 3, further comprising:
contacting the library with enzymes to nick at the deoxyuridine in the sequencing adapter;
optionally using PCR amplification to enrich for adapter-ligated fragments and to add a full sequencing adapter, and
sequencing those fragments bearing a sequencing adapter.

12. The method of claim 11, wherein the enzymes to nick the DNA comprise uracil DNA glycosylase (UDG) and/or endonuclease VIII.

13. The method of claims 2 or 3, wherein the engineered nuclease is selected from the group consisting of meganucleases, MegaTALs, zinc-finger nucleases, transcription activator effector-like nucleases (TALEN), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas RNA-guided nucleases (CRISPR/Cas RGNs), and FokI-dCas9 fusion proteins.

14. The method of claim 13, wherein the CRISPR/Cas RGN is Cas9 or Cpf1.

15. The method of claims 2 or 3, wherein treating the sample with an engineered nuclease to induce site-specific cleavage, comprises contacting the sample with a Cas9 nuclease complexed with a specific guide RNA (gRNA).

16. The method of claim 15, wherein the engineered nuclease is a Cas9 nuclease, and the method also includes utilizing a guide RNA that directs the Cas9 nuclease to a target sequence in the genome.

17. The method of claims 2 or 3, wherein the primer site comprises a next generation sequencing primer binding sequence, a randomized DNA barcode or unique molecular identifier (UMI).

18. The method of claim 1, wherein the fragments are prepared for end-ligation.

19. The method of claim 2, wherein the cleaved fragments are prepared for end-ligation.

20. The method of claim 3, further comprising end-repairing and then A-tailing of the resulting ends.

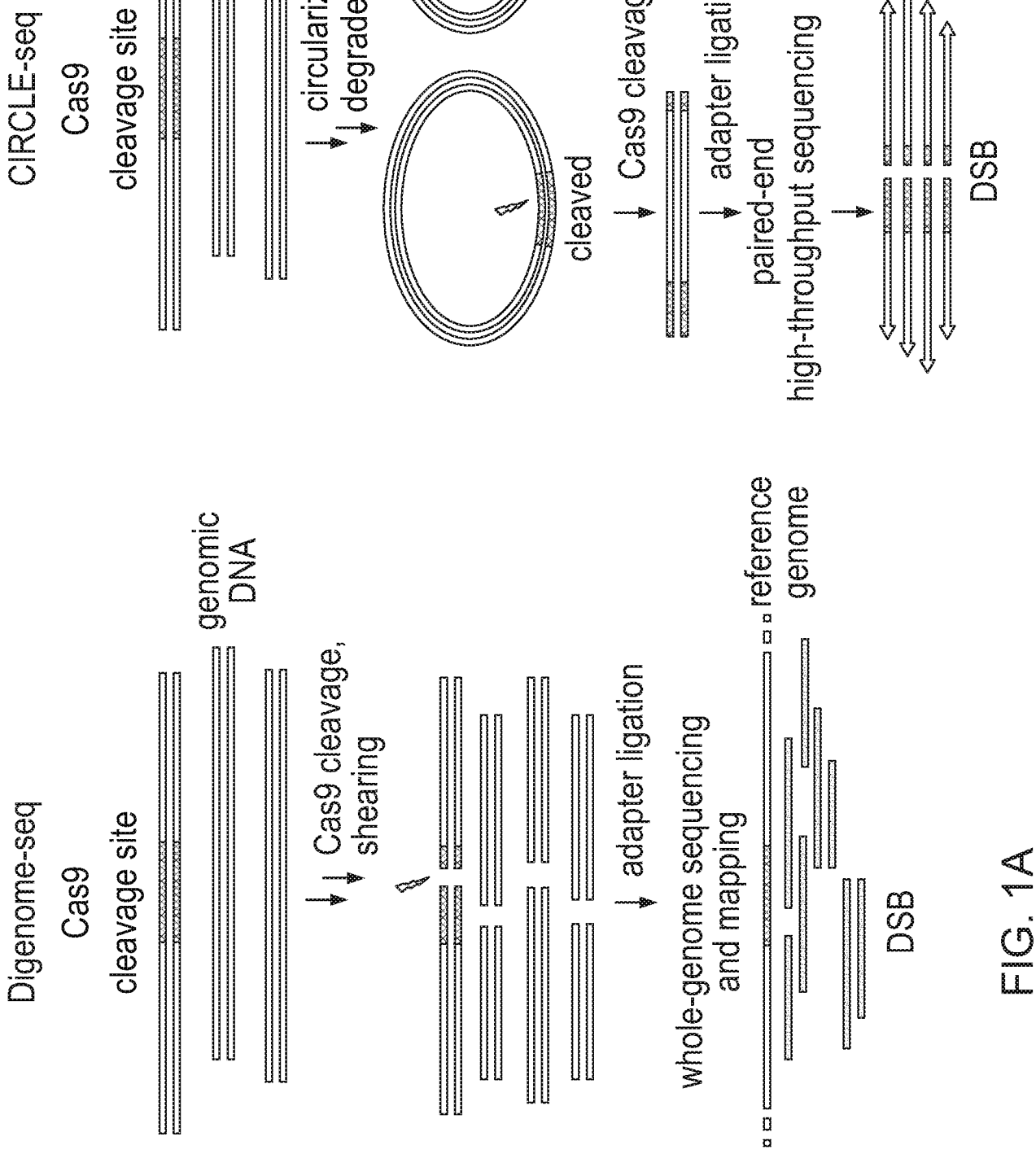

Digenome-seq
Cas9
cleavage site
genomic
DNA
Cas9 cleavage,
shearing
adapter ligation
whole-genome sequencing
and mapping
reference
genome
DSB
CIRCLE-seq
Cas9
cleavage site
sheared
genomic
DNA
circularization
degrade residual linear DNA
cleaved
uncleaved
Cas9 cleavage
adapter ligation + PCR
paired-end
high-throughput sequencing
CIRCLE-seq
reads
DSB

FIG. 1A

FIG. 1B

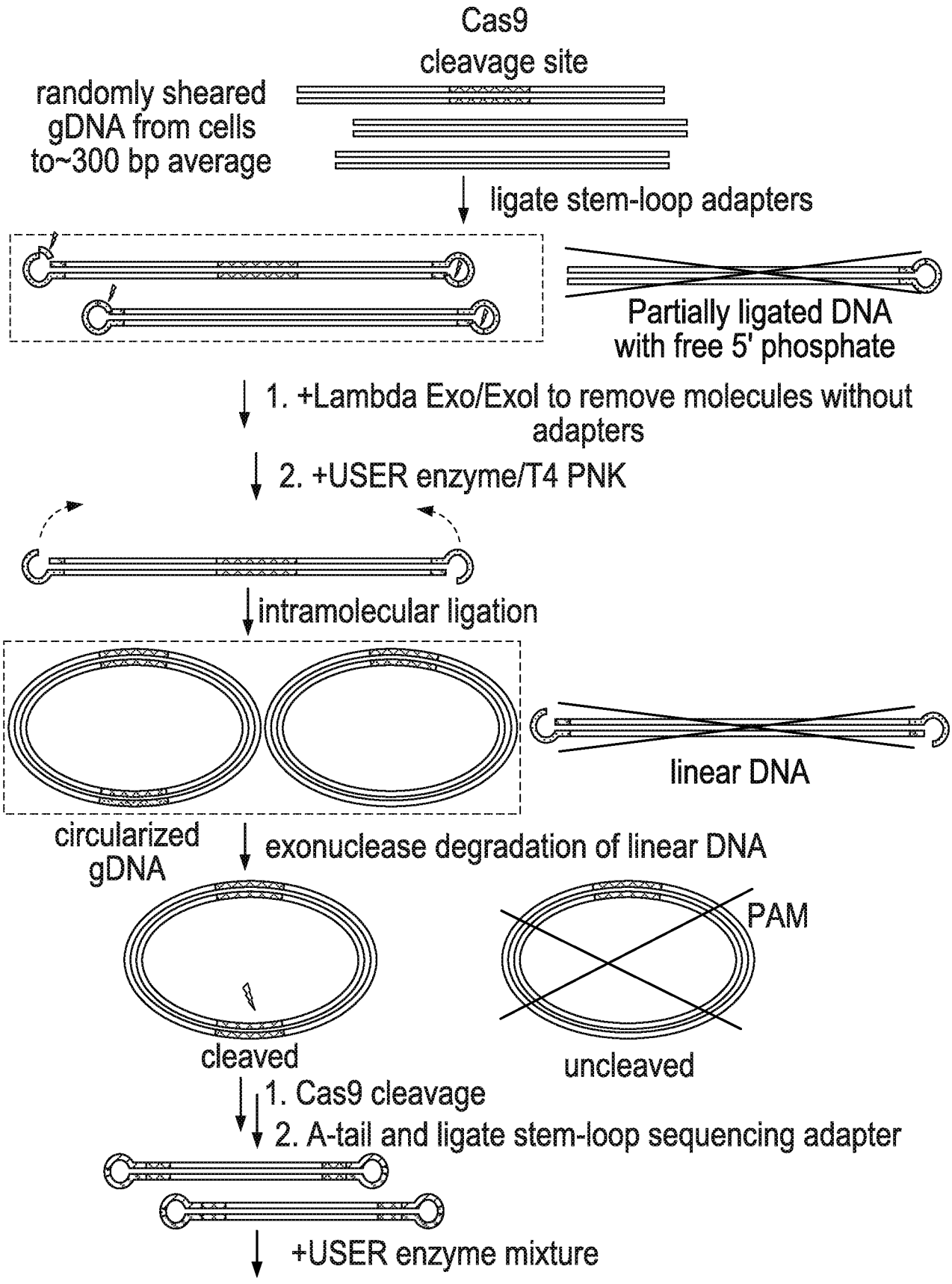

Cas9
cleavage site
randomly sheared
gDNA from cells
to~300 bp average
ligate stem-loop adapters
Partially ligated DNA
with free 5' phosphate
1. +Lambda Exo/ExoI to remove molecules without
adapters
2. +USER enzyme/T4 PNK
intramolecular ligation
linear DNA
circularized
gDNA
exonuclease degradation of linear DNA
PAM
cleaved
uncleaved
1. Cas9 cleavage
2. A-tail and ligate stem-loop sequencing adapter
+USER enzyme mixture
PCR amplification and high-throughput sequencing

FIG. 1C

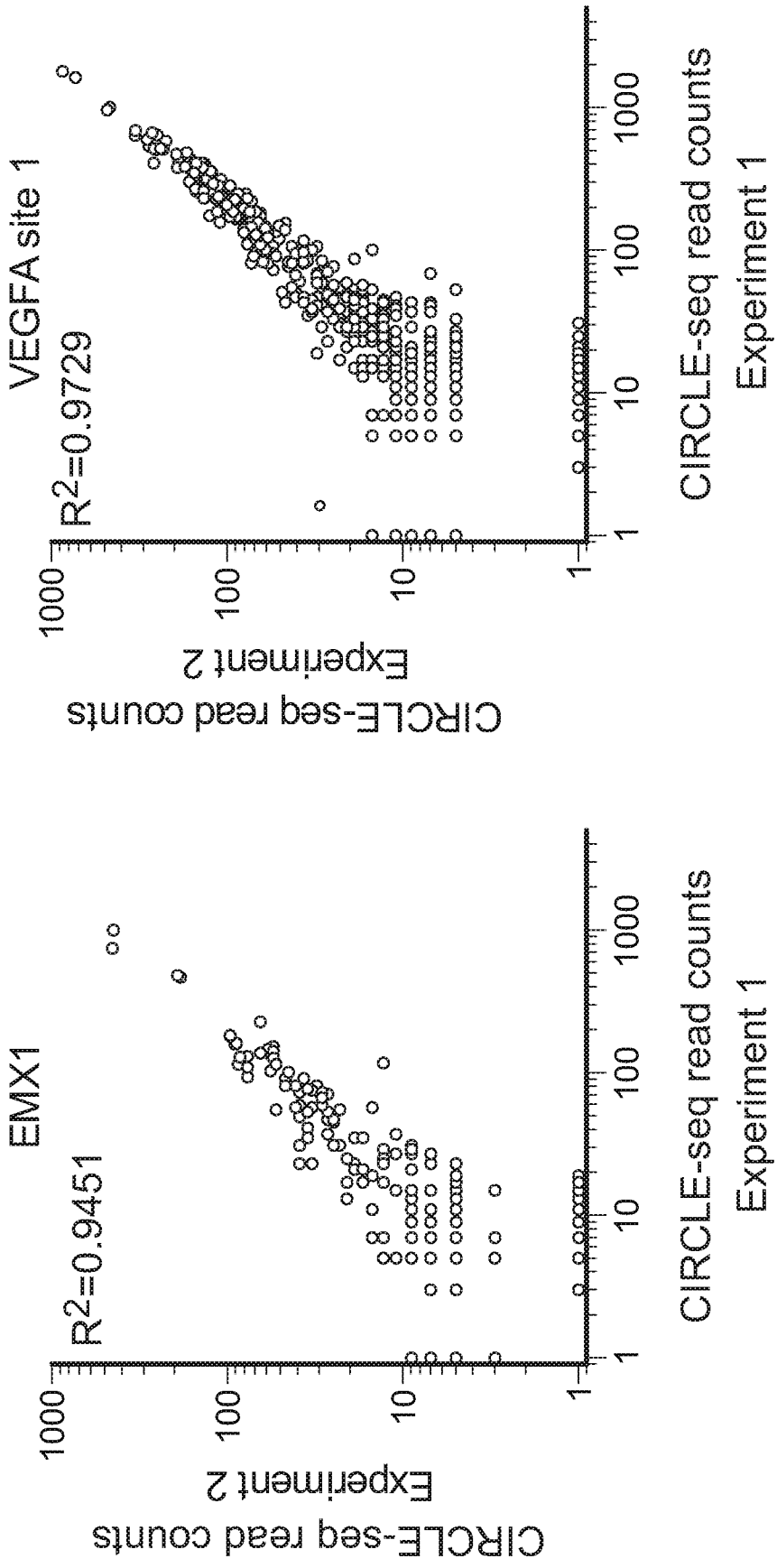

VEGFA site 1
R2=0.9729
CIRCLE-seq read counts
Experiment 1
CIRCLE-seq read counts
Experiment 2
1000
100
10
1
EMX1
R2=0.9451
CIRCLE-seq read counts
Experiment 1
CIRCLE-seq read counts
Experiment 2
1000
100
10
1

FIG. 1D

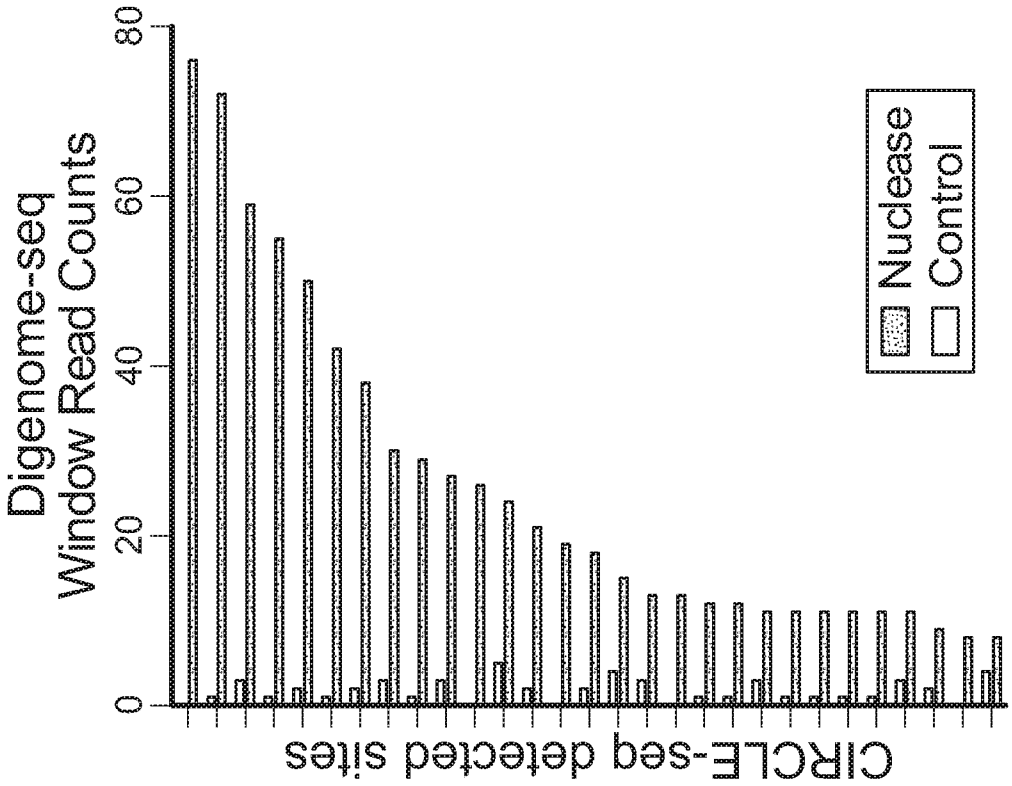

Digenome-seq
Window Read Counts
0
20
40
60
80
CIRCLE-seq detected sites
Nuclease
Control

FIG. 2B

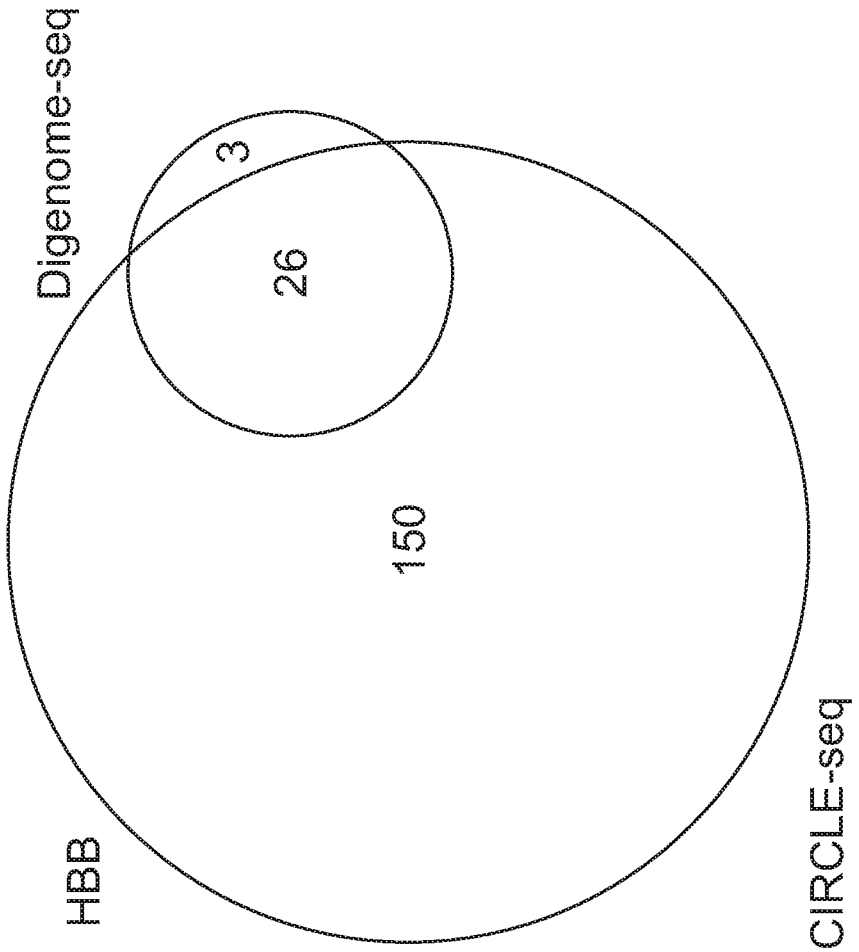

Digenome-seq
3
26
150
HBB
CIRCLE-seq

FIG. 2A

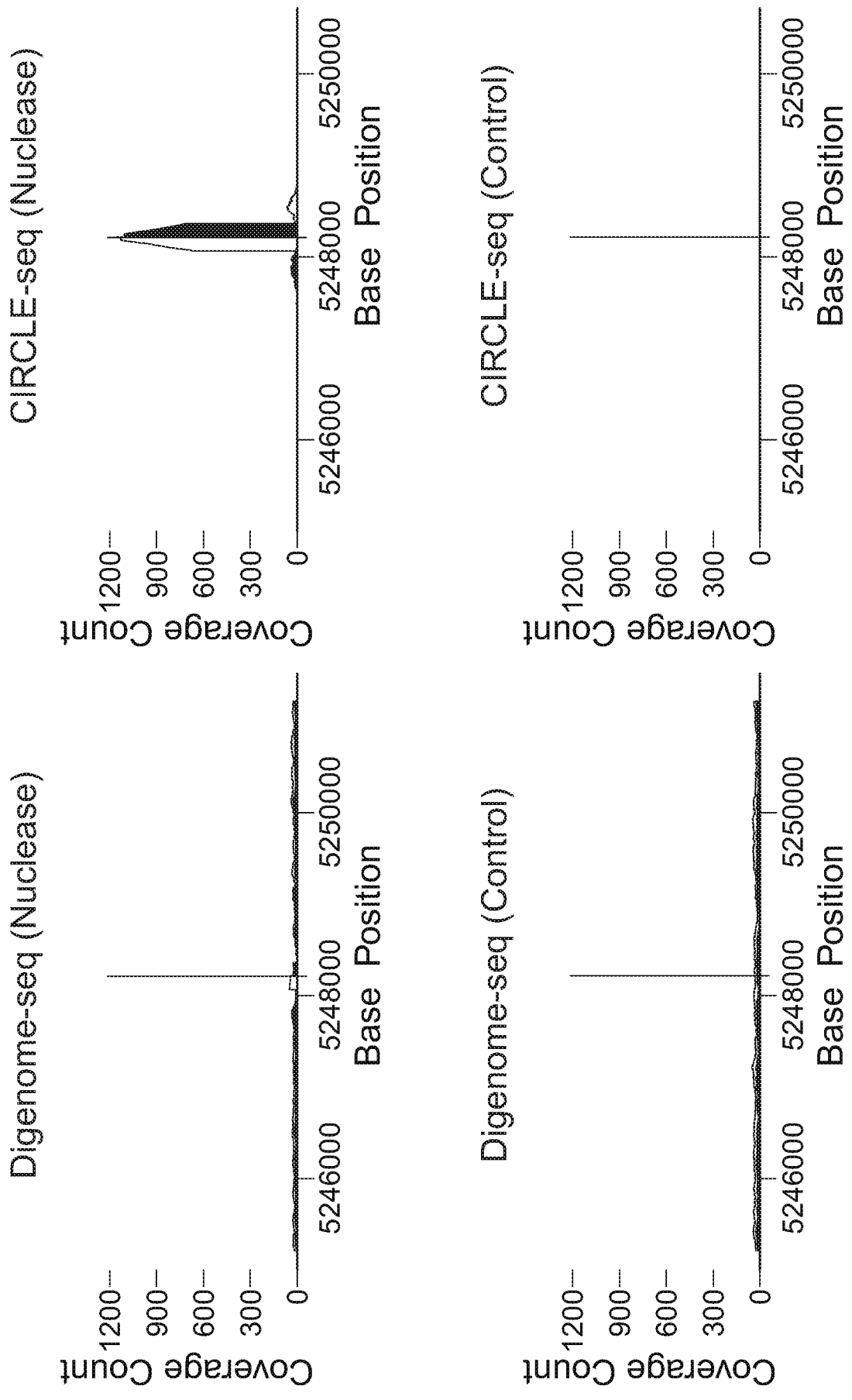

CIRCLE-seq (Nuclease)
CIRCLE-seq (Control)
Digenome-seq (Nuclease)
Digenome-seq (Control)
Coverage Count
0
300
600
900
1200
Base Position
5246000
5248000
5250000

FIG. 2C

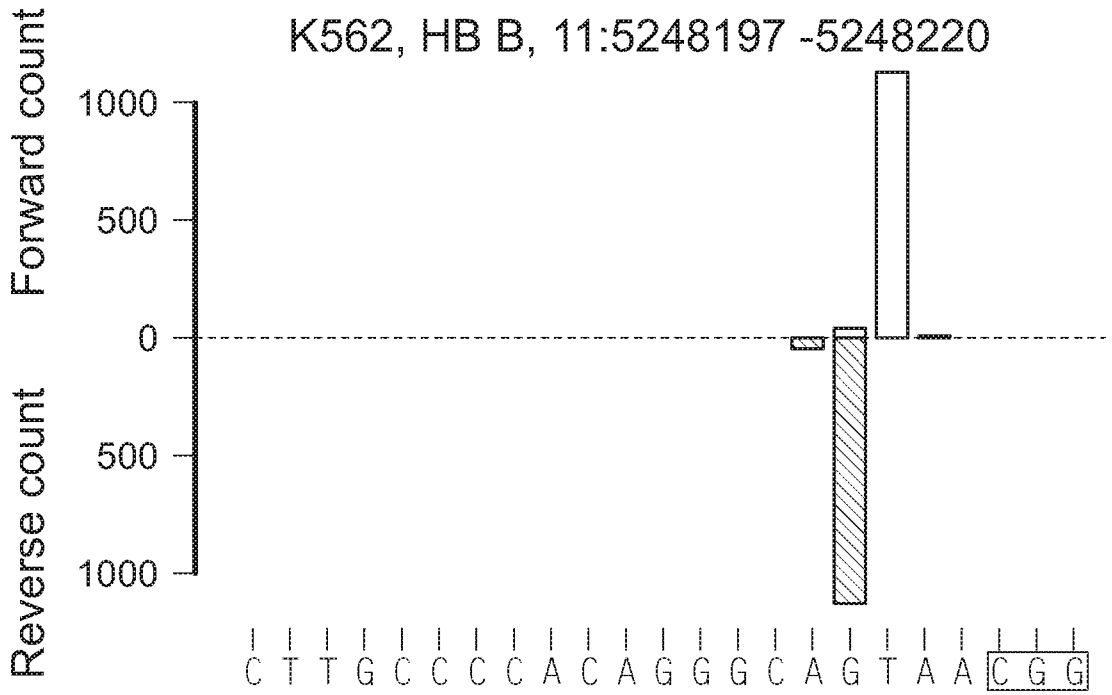

K562, HB B, 11:5248197 -5248220
Forward count
Reverse count
1000
500
0
500
1000
C T T G C C C C A C A G G G C A G T A A C G G

FIG. 2D

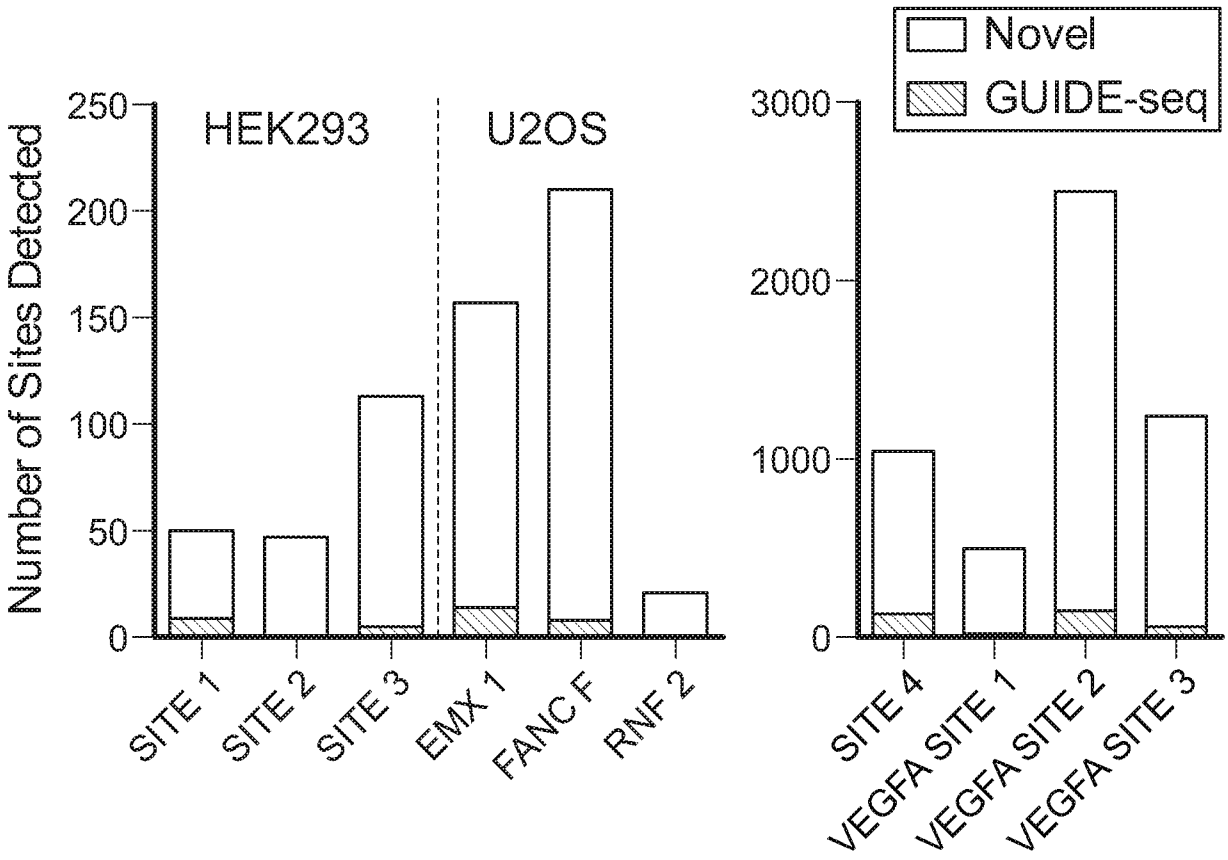

Novel
GUIDE-seq
Number of Sites Detected
HEK293
U2OS
250
200
150
100
50
0
3000
2000
1000
0
SITE 1
SITE 2
SITE 3
EMX 1
FANCF
RNF 2
SITE 4
VEGFA SITE 1
VEGFA SITE 2
VEGFA SITE 3

FIG. 3A

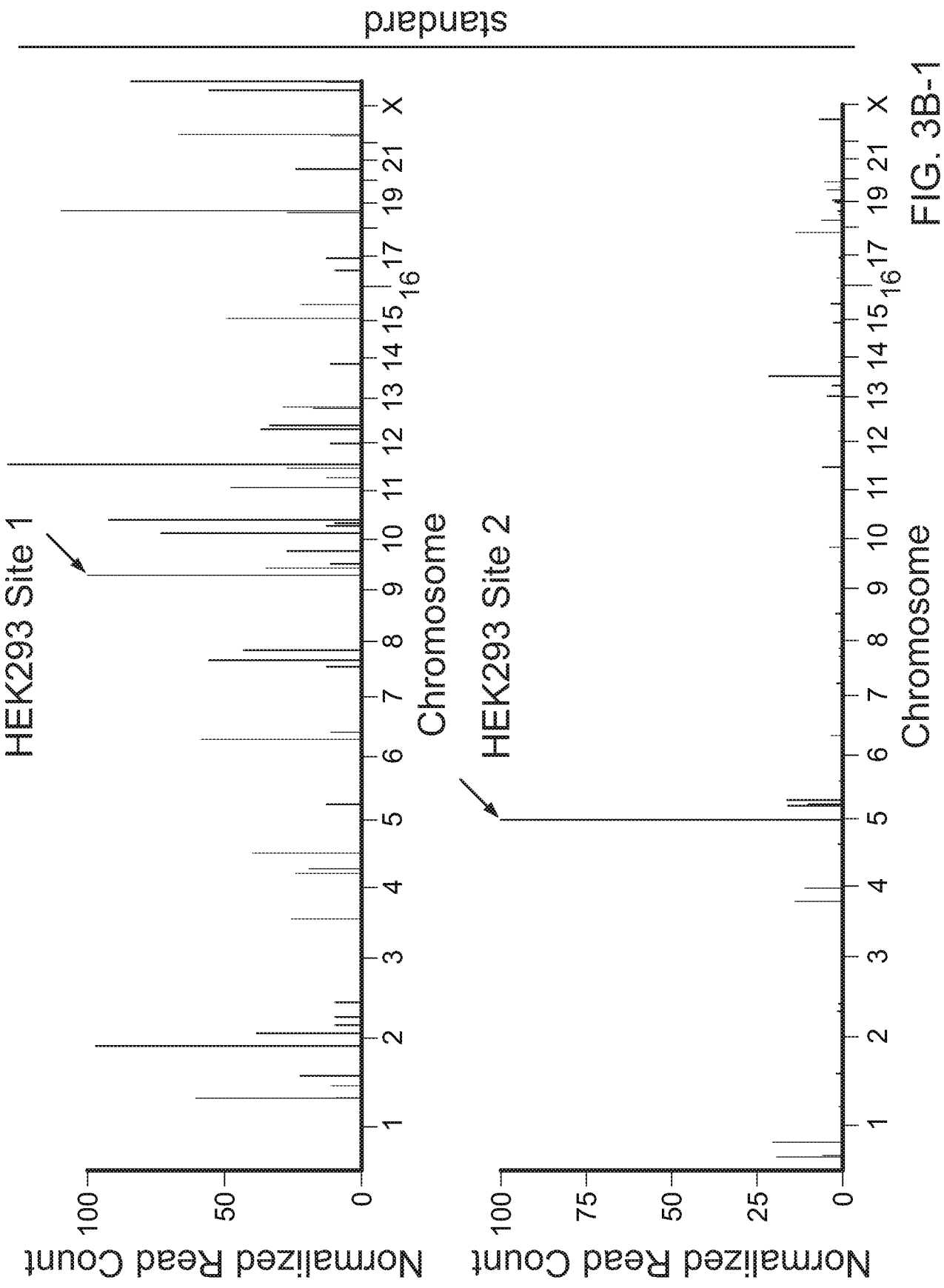

standard
HEK293 Site 1
HEK293 Site 2
Normalized Read Count
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
19
21
X
0
25
50
75
100

FIG. 3B-1

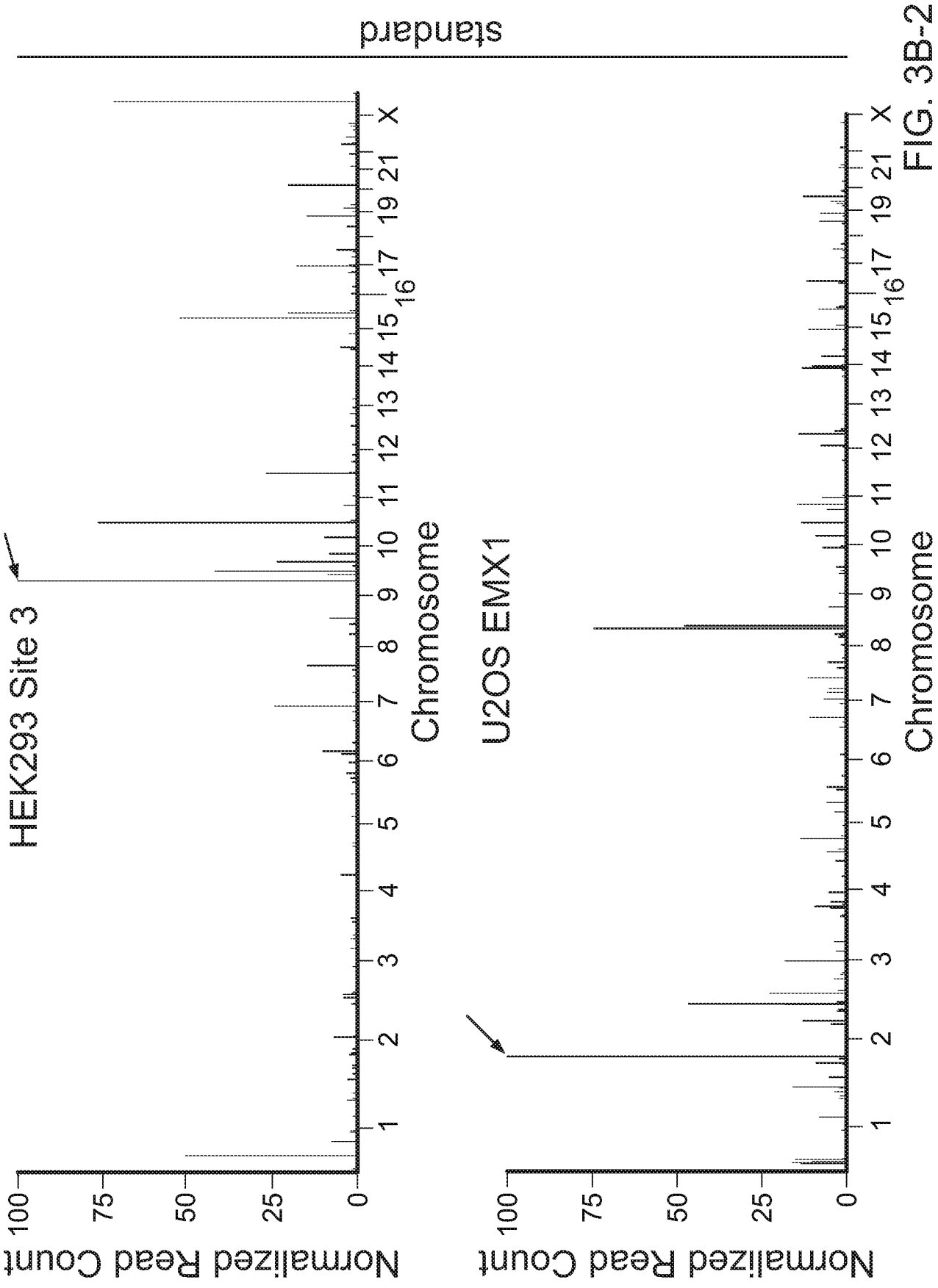

standard
HEK293 Site 3
U2OS EMX1
Normalized Read Count
100
75
50
25
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
19
21
X
Chromosome

FIG. 3B-2

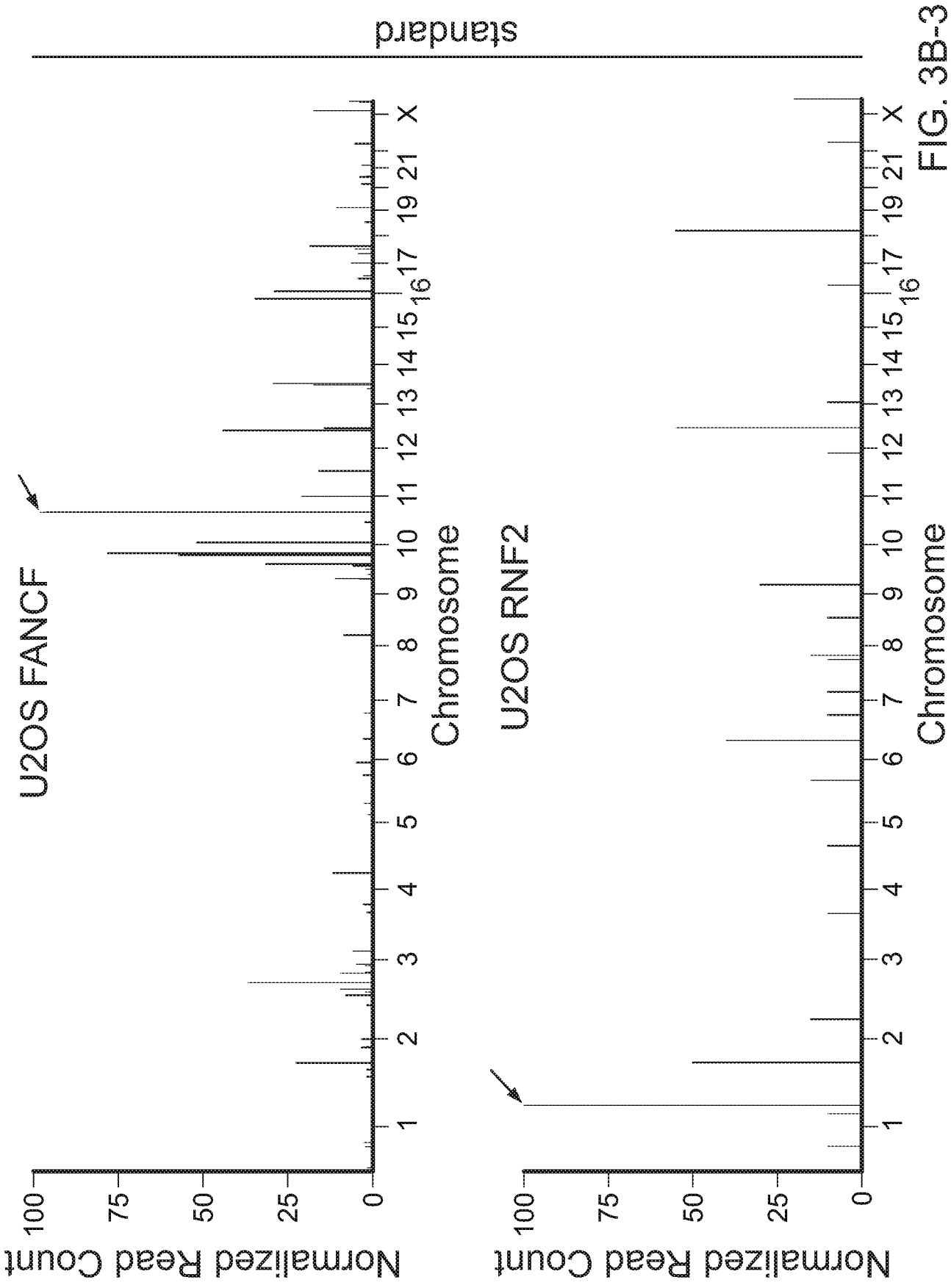

standard
U2OS FANCF
U2OS RNF2
Normalized Read Count
Chromosome
0
25
50
75
100
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
19
21
X

FIG. 3B-3

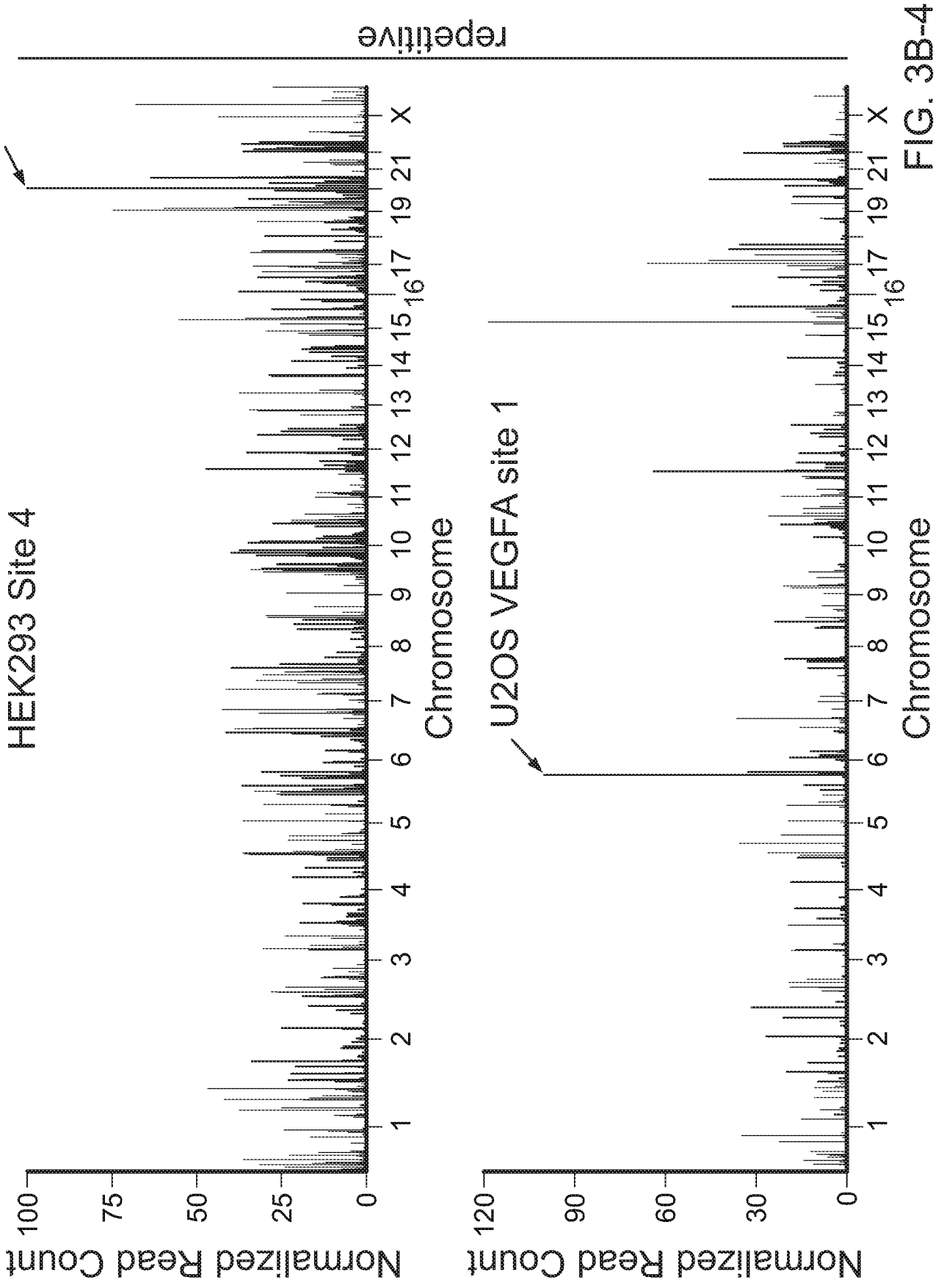

repetitive
HEK293 Site 4
Normalized Read Count
100
75
50
25
0
Chromosome
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 19 21 X
U2OS VEGFA site 1
Normalized Read Count
120
90
60
30
0
Chromosome
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 19 21 X

FIG. 3B-4

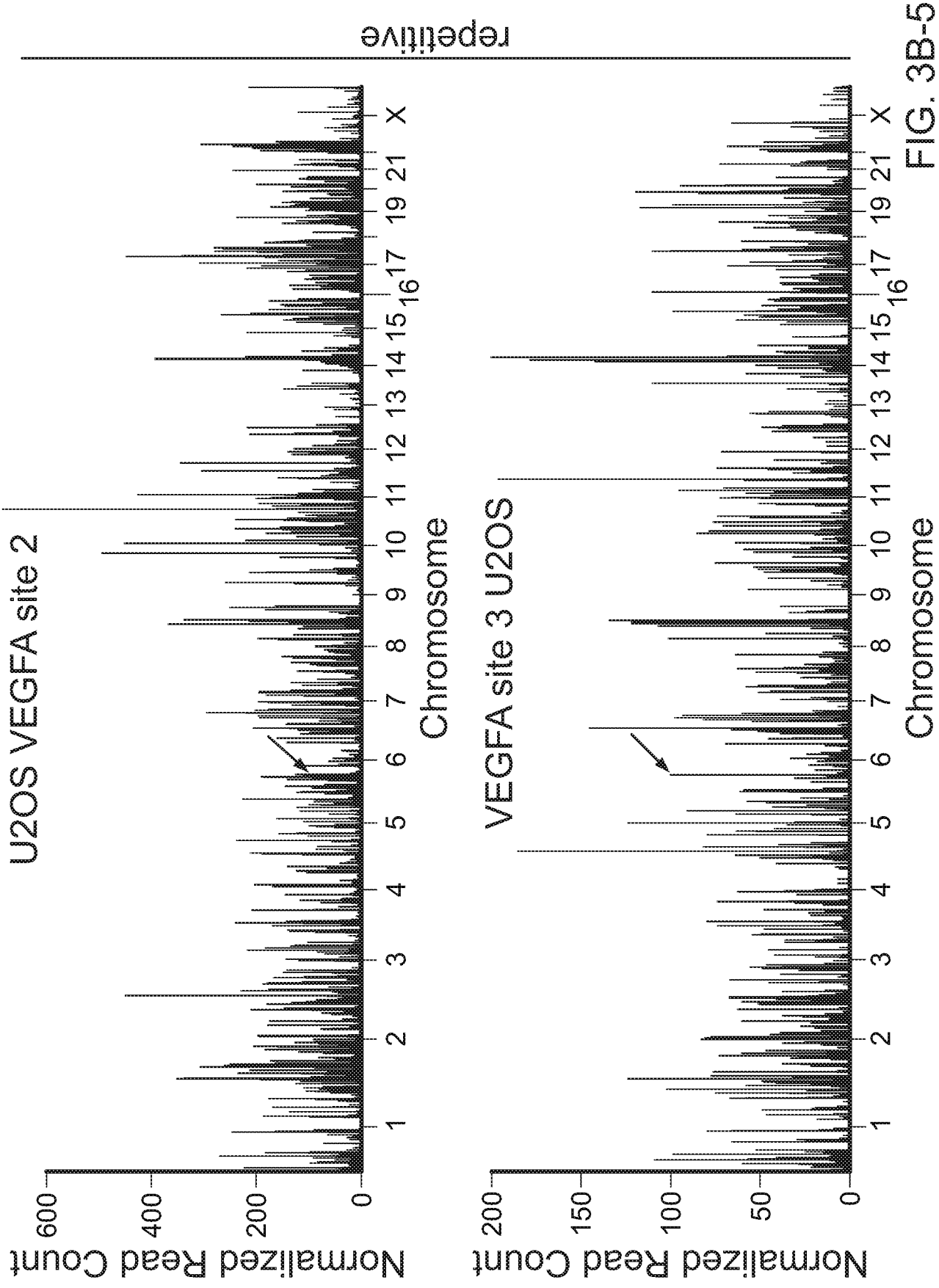

repetitive
U2OS VEGFA site 2
Normalized Read Count
0
200
400
600
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
19
21
X
VEGFA site 3 U2OS
Normalized Read Count
0
50
100
150
200
Chromosome

FIG. 3B-5

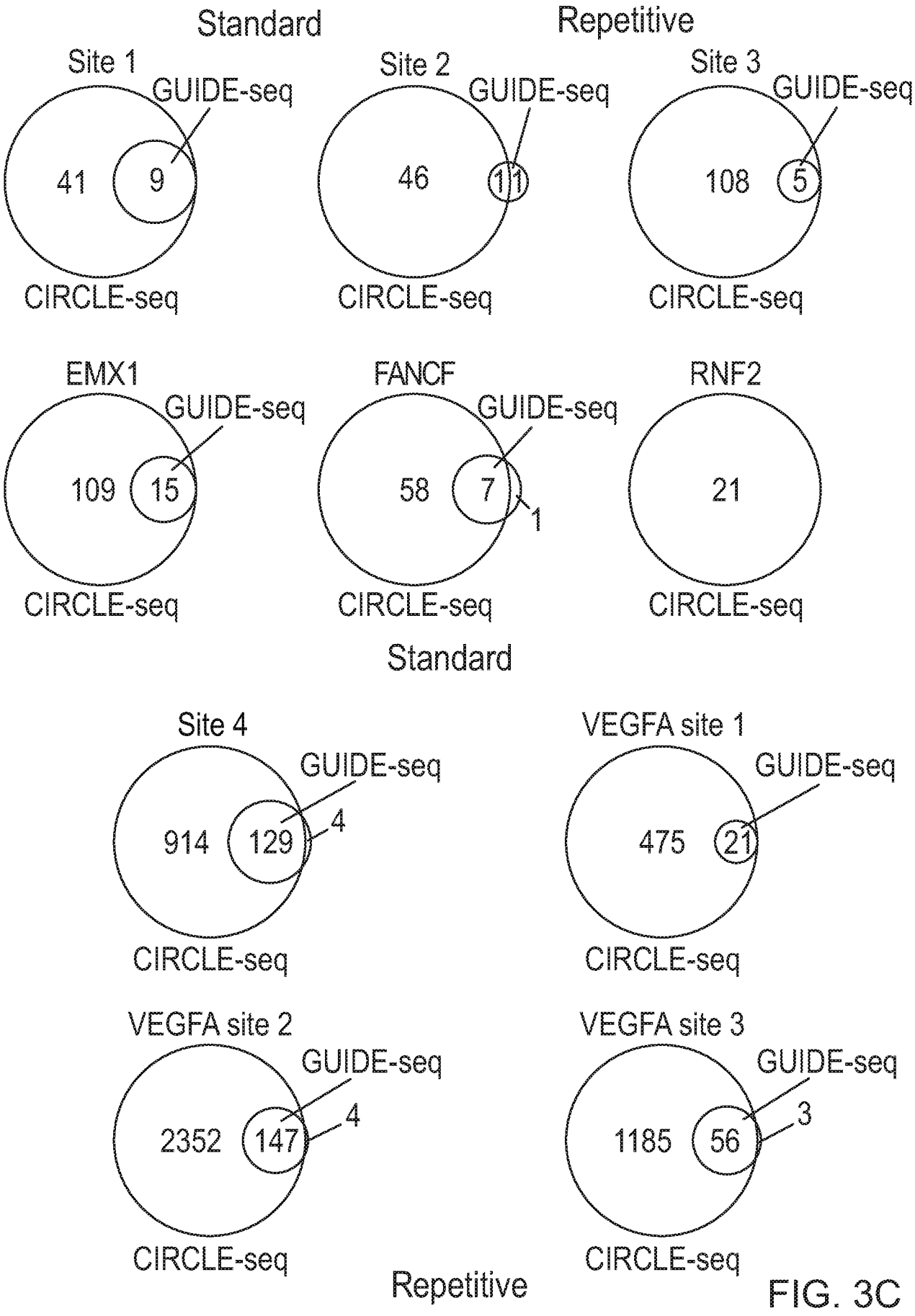

Standard
Repetitive
Site 1
GUIDE-seq
41
9
CIRCLE-seq
Site 2
GUIDE-seq
46
11
CIRCLE-seq
Site 3
GUIDE-seq
108
5
CIRCLE-seq
EMX1
GUIDE-seq
109
15
CIRCLE-seq
FANCF
GUIDE-seq
58
7
1
CIRCLE-seq
RNF2
21
CIRCLE-seq
Standard
Site 4
GUIDE-seq
914
129
4
CIRCLE-seq
VEGFA site 1
GUIDE-seq
475
21
CIRCLE-seq
VEGFA site 2
GUIDE-seq
2352
147
4
CIRCLE-seq
VEGFA site 3
GUIDE-seq
1185
56
3
CIRCLE-seq
Repetitive

FIG. 3C

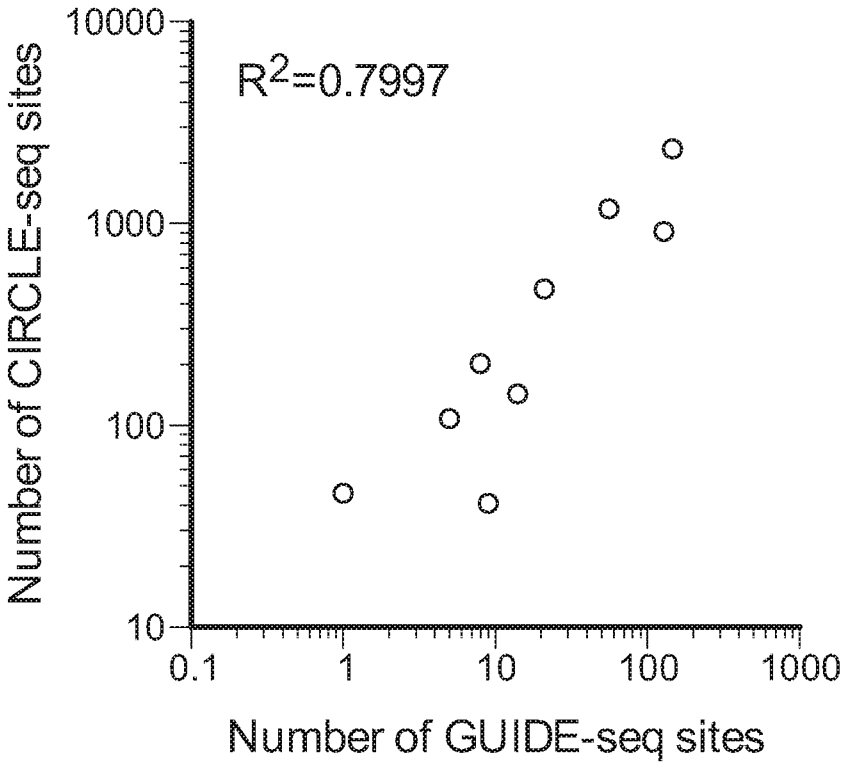

10000
1000
100
10
R^2=0.7997
Number of CIRCLE-seq sites
0.1
1
10
100
1000
Number of GUIDE-seq sites

FIG. 3D

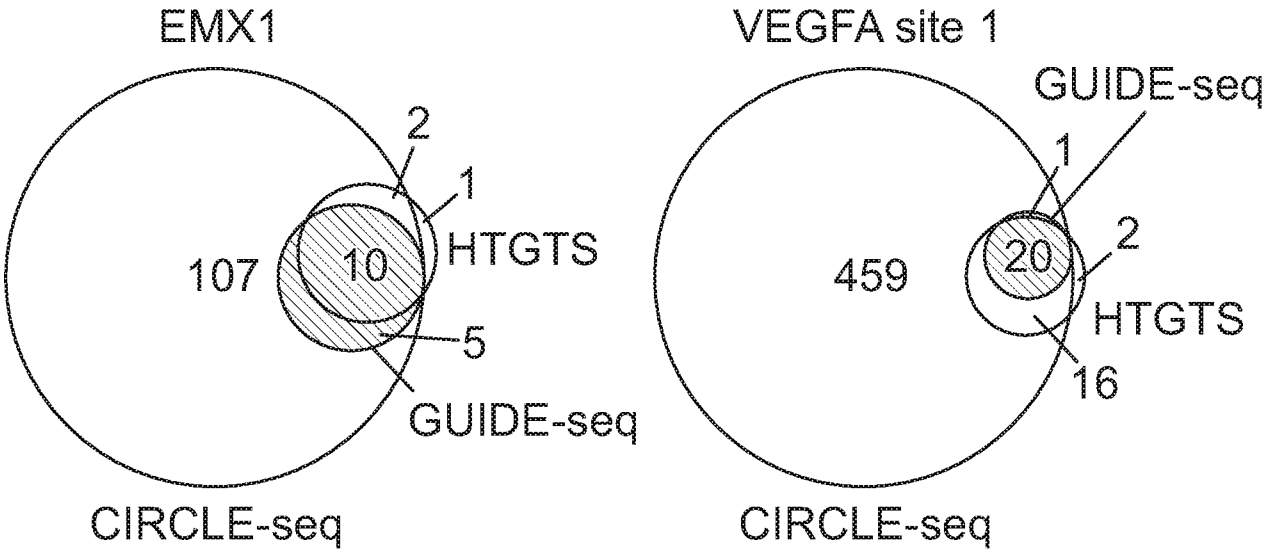

EMX1
2
1
107
10
HTGTS
5
GUIDE-seq
CIRCLE-seq
VEGFA site 1
GUIDE-seq
1
459
20
2
HTGTS
16
CIRCLE-seq

FIG. 3E

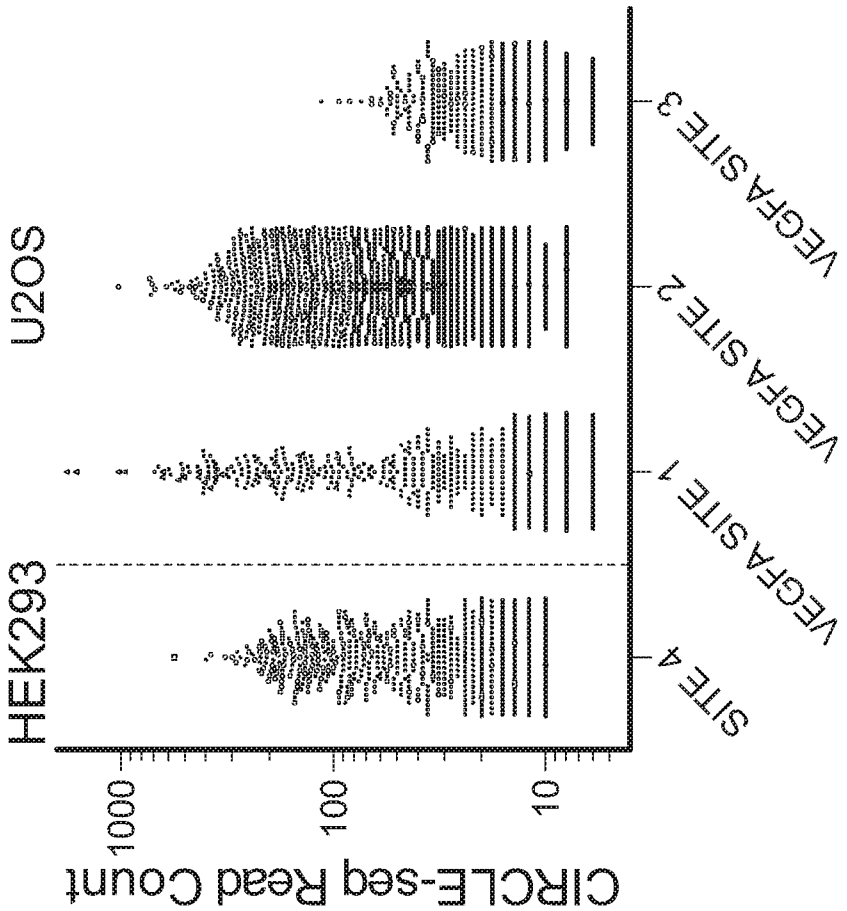
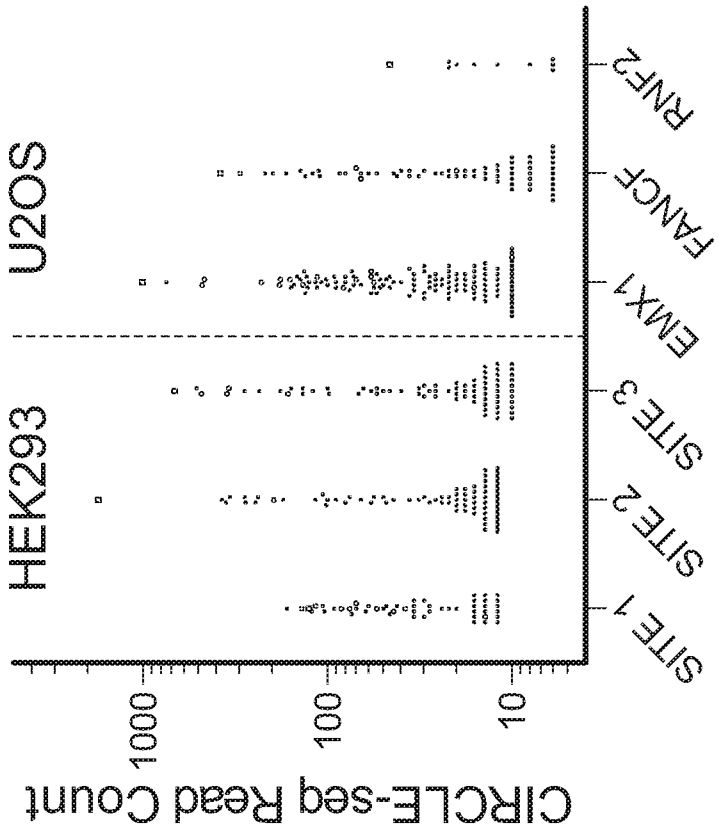

HEK293
U2OS
CIRCLE-seq Read Count
1000
100
10
SITE 4
VEGFA SITE 1
VEGFA SITE 2
VEGFA SITE 3

FIG. 4A


HEK293
U2OS
CIRCLE-seq Read Count
1000
100
10
SITE 1
SITE 2
SITE 3
EMX1
FANCF
RNF2

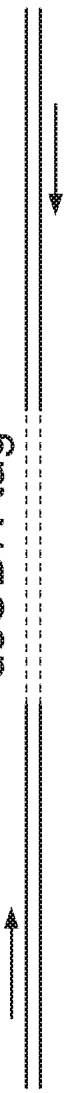

dsODN tag
Targeted Tag Sequencing

FIG. 4B

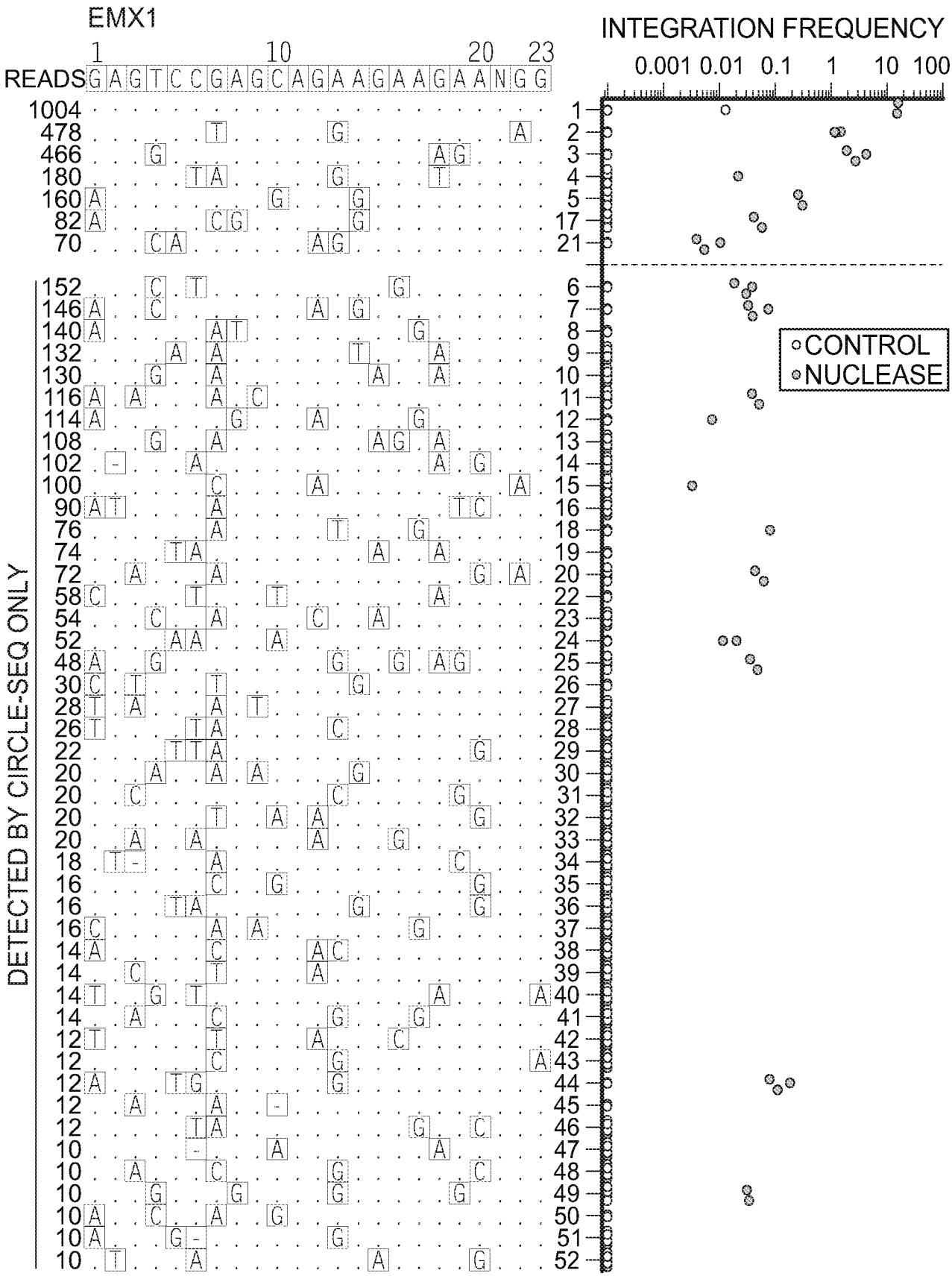

EMX1
1
10
20
23
READS
G A G T C C G A G C A G A A G A A G A A N G G
INTEGRATION FREQUENCY
0.001 0.01 0.1 1 10 100
CONTROL
NUCLEASE
DETECTED BY CIRCLE-SEQ ONLY

FIG. 4C

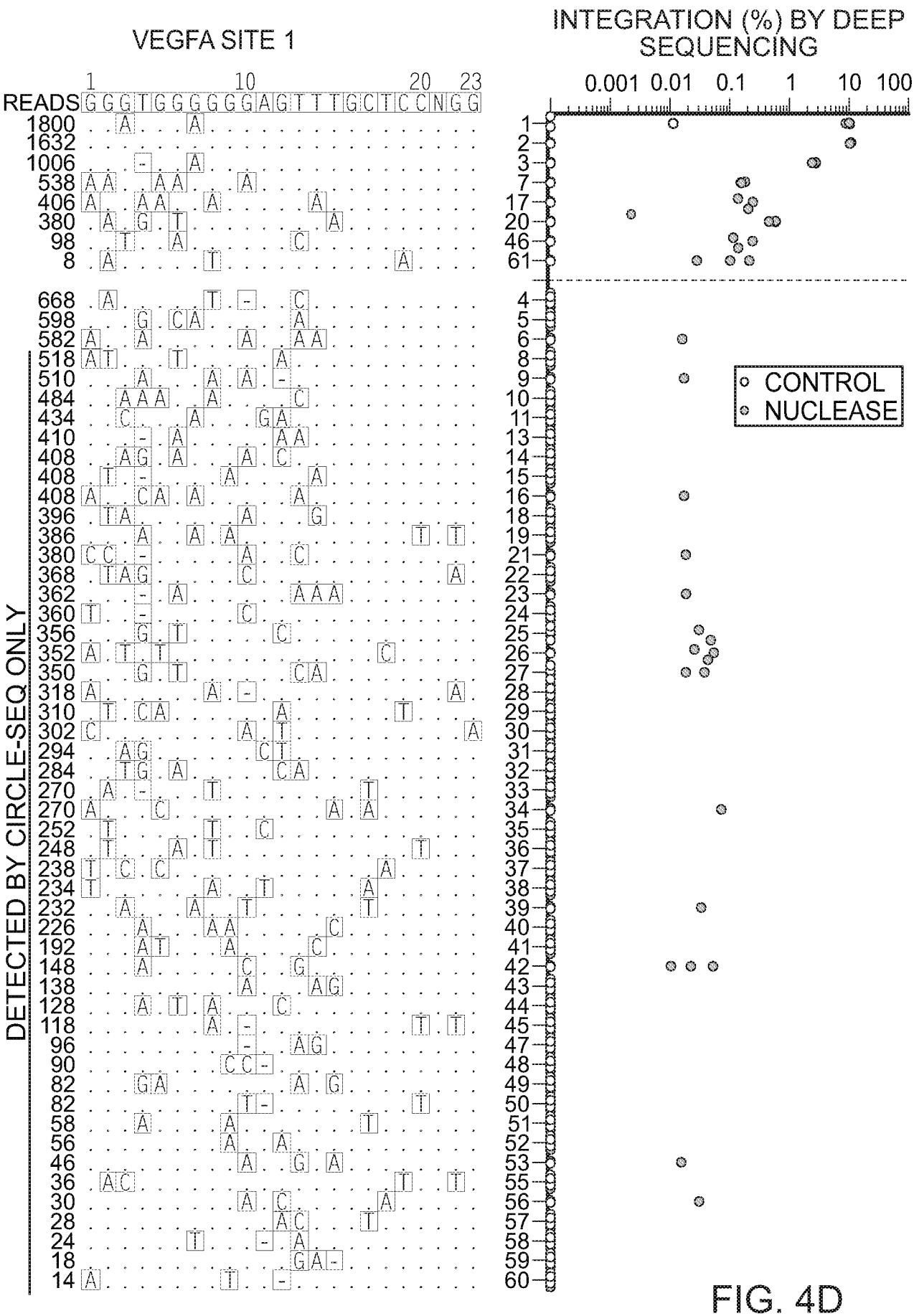

VEGFA SITE 1
INTEGRATION (%) BY DEEP SEQUENCING
0.001 0.01 0.1 1 10 100
CONTROL
NUCLEASE
DETECTED BY CIRCLE-SEQ ONLY

FIG. 4D

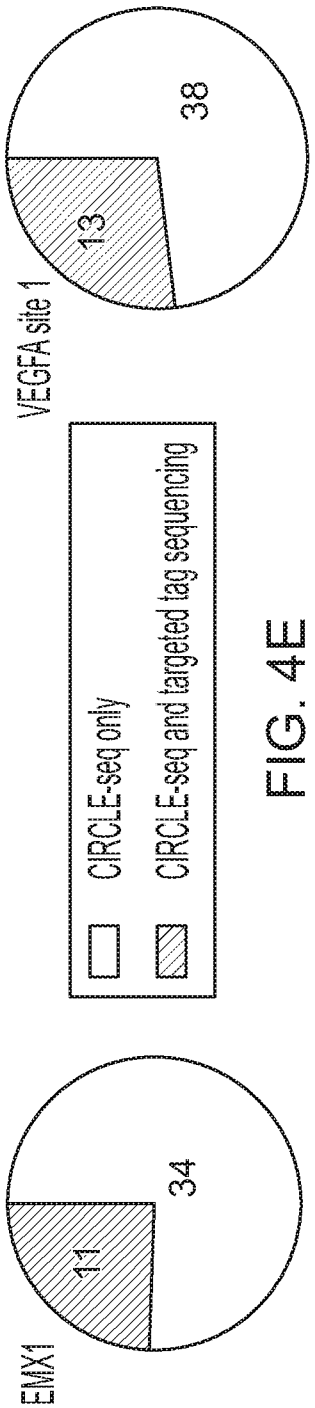

EMX1
11
34
VEGFA site 1
13
38
CIRCLE-seq only
CIRCLE-seq and targeted tag sequencing

FIG. 4E

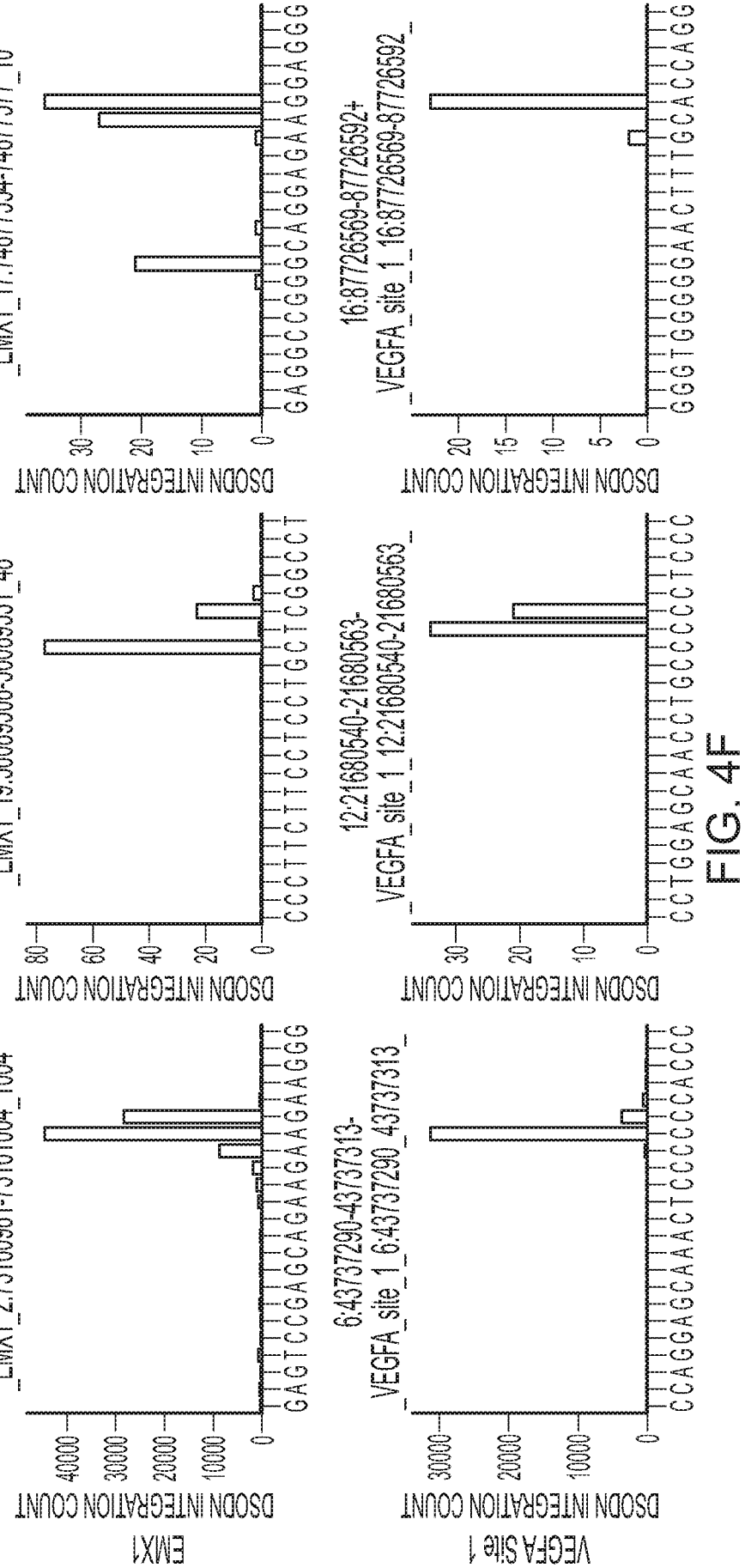
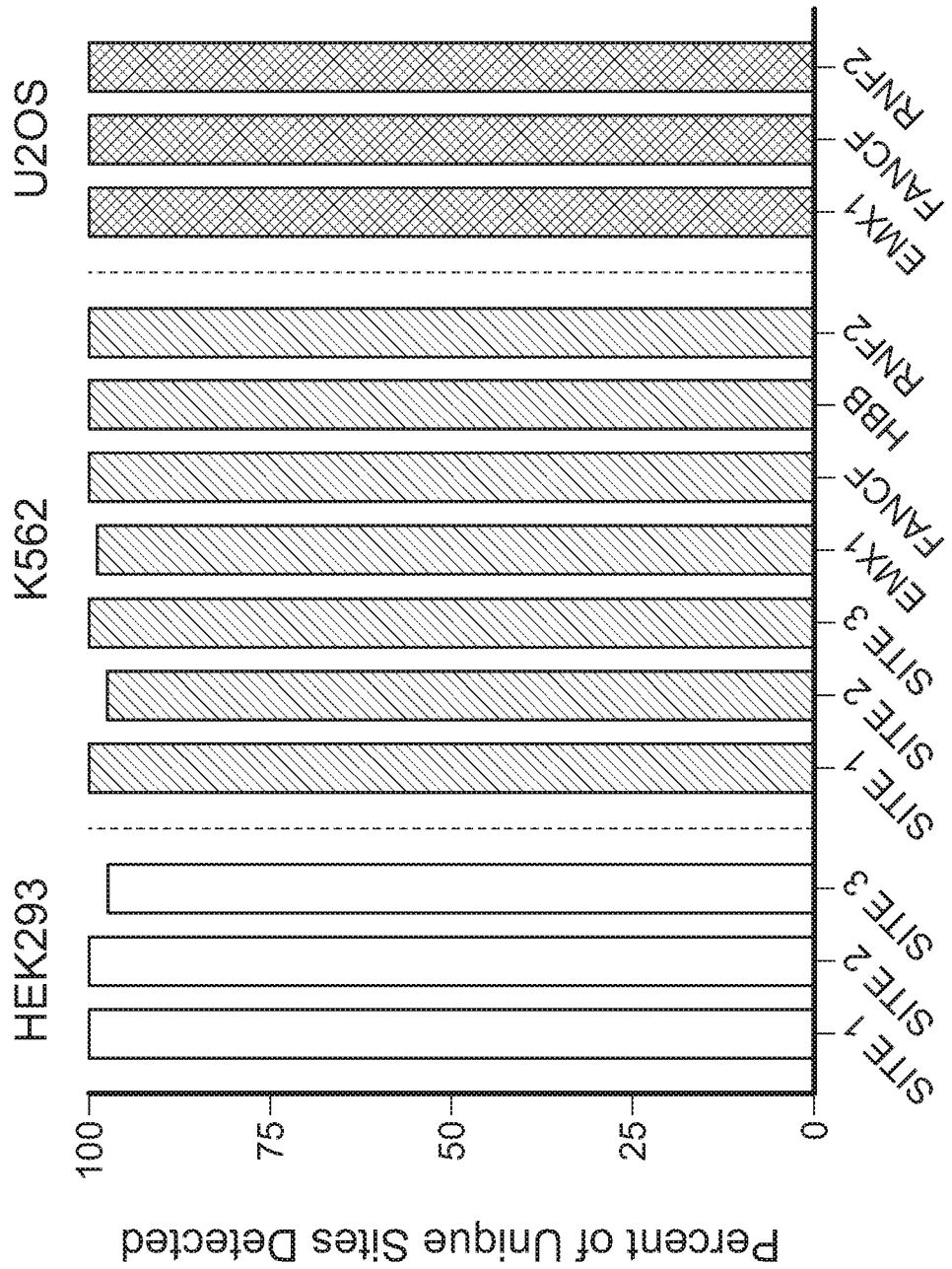

2:73160981-73161004+
EMX1_2:73160981-73161004_1004
19:56089508-56089531-
EMX1_19:56089508-56089531_48
6:43737290-43737313-
VEGFA_site_1_6:43737290_43737313_
12:21680540-21680563-
VEGFA_site_1_12:21680540-21680563_
17:74877554-74877577+
EMX1_17:74877554-74877577_10
16:87726569-87726592+
VEGFA_site_1_16:87726569-87726592_
DSODN INTEGRATION COUNT
EMX1
VEGFA Site 1

FIG. 4F



Supplementary Figure 7. Percentage of unique cle

FIG. 4G

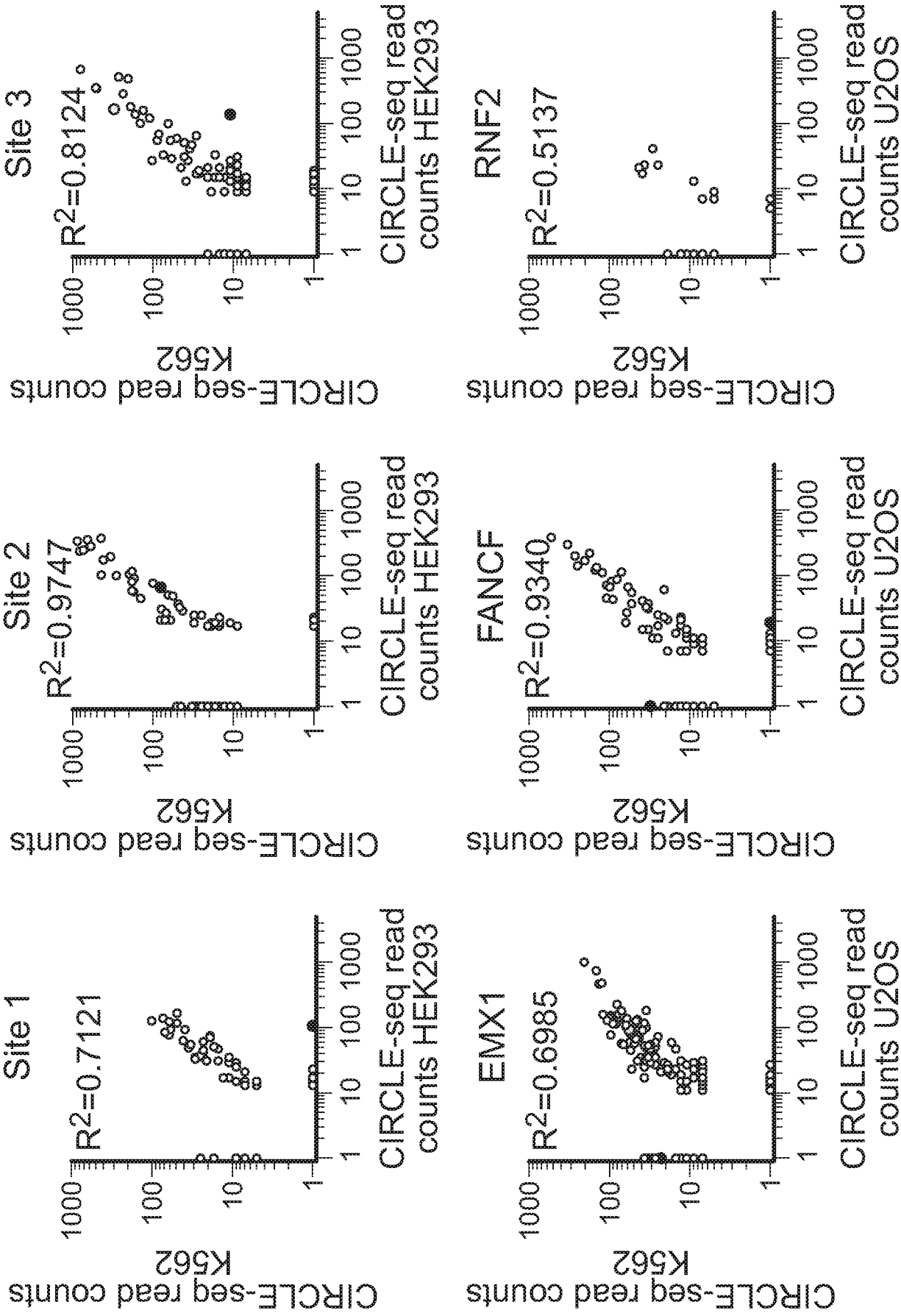

Site 1
R2=0.7121
Site 2
R2=0.9747
Site 3
R2=0.8124
EMX1
R2=0.6985
FANCF
R2=0.9340
RNF2
R2=0.5137
CIRCLE-seq read counts K562
CIRCLE-seq read counts HEK293
CIRCLE-seq read counts U2OS
1000
100
10
1
1 10 100 1000

FIG. 5A

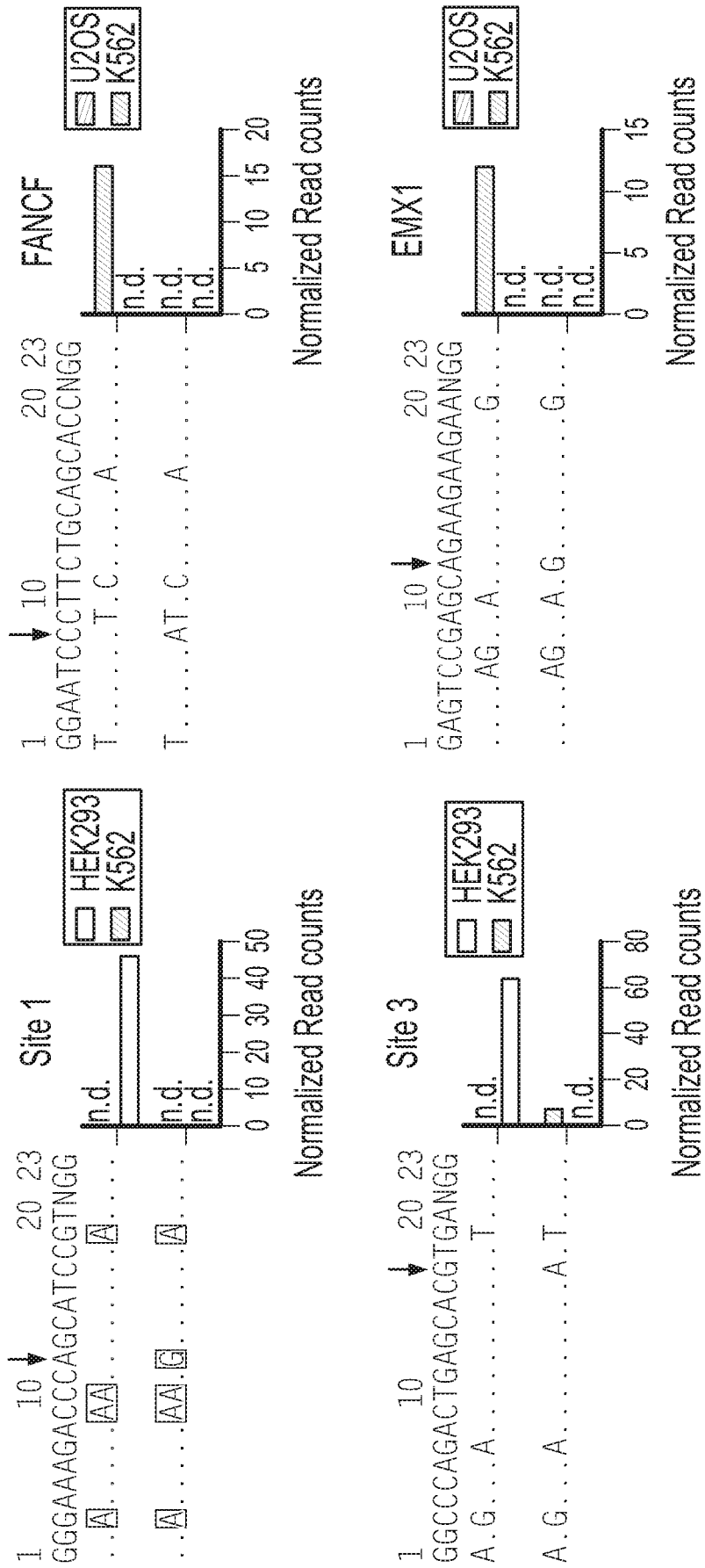

Site 1
HEK293
K562
1 10 20 23
GGGAAAGACCCAGCATCCGTNGG
n.d.
n.d.
n.d.
0 10 20 30 40 50
Normalized Read counts
FANCF
U2OS
K562
1 10 20 23
GGAATCCCTTCTGCAGCACCNGG
n.d.
n.d.
n.d.
0 5 10 15 20
Normalized Read counts
Site 3
HEK293
K562
1 10 20 23
GGCCCAGACTGAGCACGTGANGG
n.d.
n.d.
0 20 40 60 80
Normalized Read counts
EMX1
U2OS
K562
1 10 20 23
GAGTCCGAGCAGAAGAAGANGG
n.d.
n.d.
n.d.
0 5 10 15
Normalized Read counts

FIG. 5B

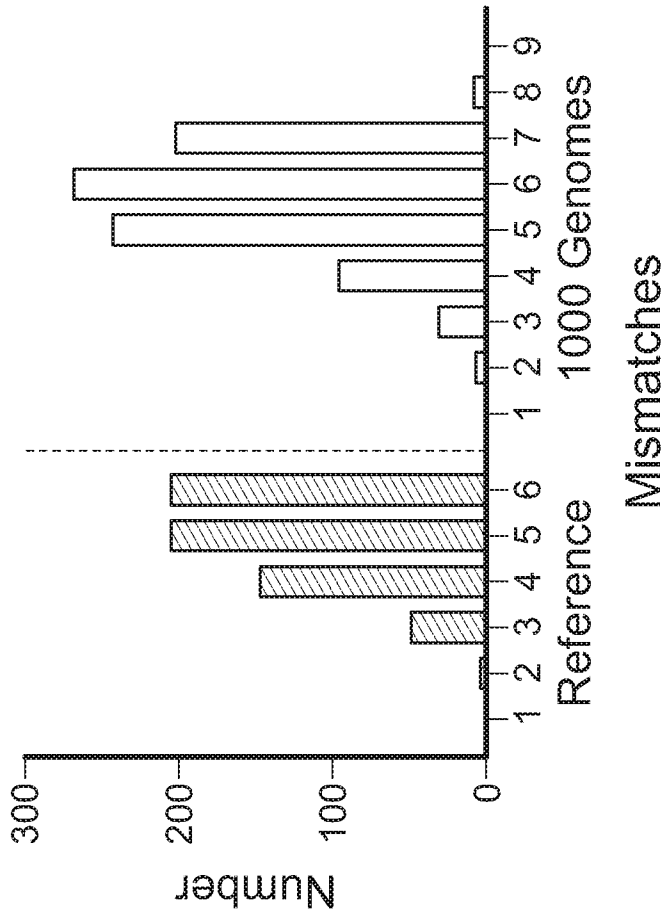


FIG. 5D

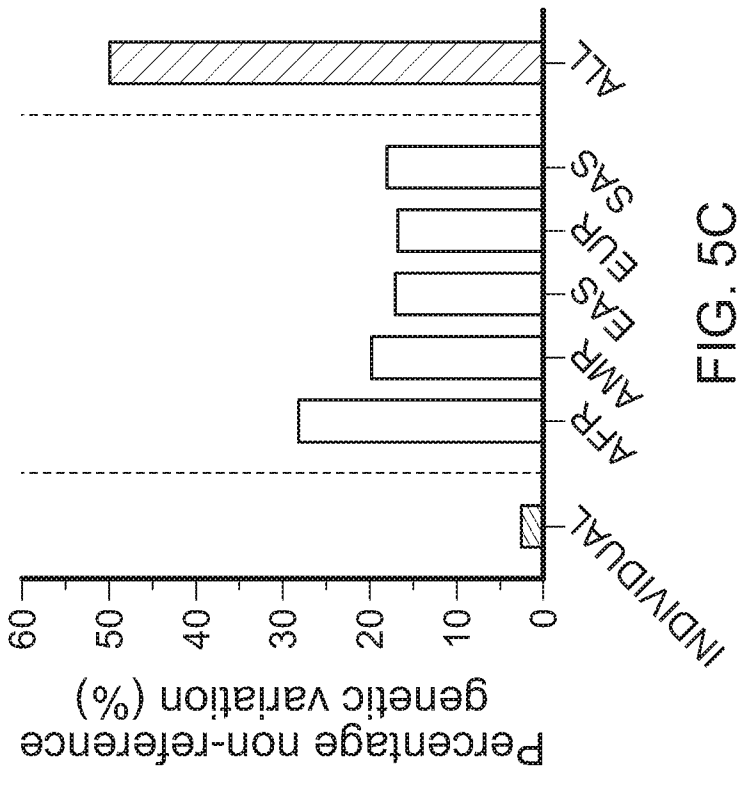


FIG. 5C

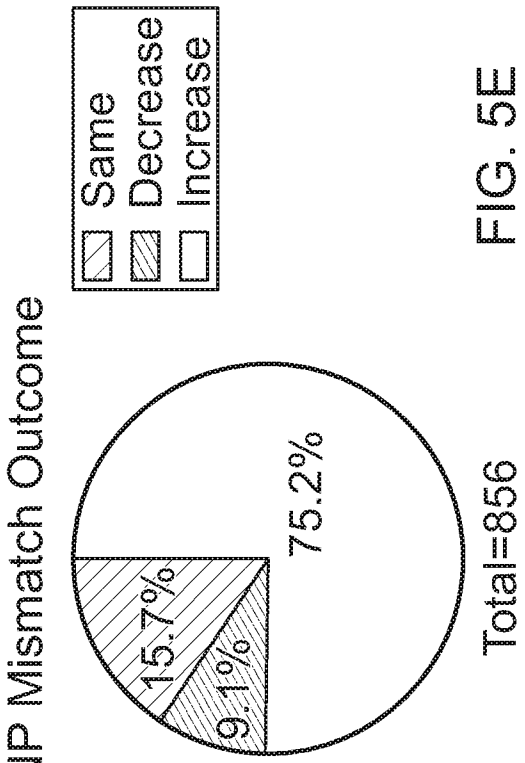


FIG. 5E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/54912

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/54912

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-8
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/54912

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/10, C12Q 1/68 (2016.01)
CPC - C12N 15/1093, C12Q 1/6869, C40B 40/06
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12N 15/10, C12Q 1/68 (2016.01)
CPC - C12N 15/1093, C12Q 1/6869, C40B 40/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12N 15/1065
(keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar
Search terms: double-stranded DNA, dsDNA, CRISPR, Cas9, random, shear, cleavage, deoxyuridine, stem, loop, stem-loop, ligate, adapter, library

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/0137605 A1 (UNIVERSITY OF WASHINGTON) 30 May 2013 (30.05.2013) para [0005], [0007], [0076], [0077], [0080], [0088], [0093], [0103], [0152], [0158], [0186]	1-3, 9-20
Y	US 2013/0309668 A1 (RUBICON GENOMICS INC.) 21 November 2013 (21.11.2013) para [0064], [0074], [0081], [0105], [0203], [0328], [0330], [0331], [0417], [0429]	1-3, 9-20
Y	US 2014/0295557 A1 (THE GENERAL HOSPITAL CORPORATION) 2 October 2014 (02.10.2014) para [0005], [0009], [0011], [0050], [0105], [0186]	2-3, 9-17, 19-20

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 January 2017	24 JAN 2017

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774