Analytical Biochemistry **304,** 206–211 (2002)
doi:10.1006/abio.2002.5634, available online at http://www.idealibrary.com on IDEAL®

Quality and Quantity of DNA Isolated from Frozen Urine in Population-Based Research

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Received October 29, 2001

In several population-based studies in the past urine samples were collected and stored for future research. We set out to determine the reliability of using such samples for genotyping DNA markers in epidemiologic research. A source of DNA extracted from exfoliated nucleated cells in urine is provided by the DOM cohort, in which specimens were collected 15–25 years ago. We have examined the quality of the DNA in 48 of these samples by measuring the amount of DNA isolated and its ability to provide an adequate PCR template for amplicons of different lengths. MTHFR polymorphism was analyzed in 644 specimens to determine the inter- and intraobserver reproducibility. Although the DNA amount was variable, 26 to 89% of the samples, depending both on the length of the PCR amplicon and on PCR conditions, yielded a visible PCR product. The intra- and interobserver agreements were comparable (κ 0.86 and 0.88, respectively). Our results demonstrate that frozen urine samples can be used for DNA typing studies in women after prolonged periods of storage, but with sometimes unpredictable results. Ultimately, the genotype success rate was 89.3%. Urine collection can be considered as a useful method of obtaining DNA in large cohort studies and other circumstances when blood samples cannot be obtained or have not been stored.

Key Words: **DNA isolation; PCR; storage; urine.**

Genetic epidemiology is a relatively new discipline that attempts to combine the methods of epidemiology, and of human population and molecular genetics to study complex diseases, in which predisposing genetic markers are believed to play a role. In several prospective studies, biobanks have been used to store human tissues such as blood, urine, hair, and nails of study subjects. Following a follow-up period, samples of various disease groups and control groups can be constructed from the original cohort (nested case control studies). The biological specimens can then be recovered from storage and used for either protein analysis for hormone and serum protein determinations or for investigating the possible role of gene variations at DNA sequence level. There may be restrictions on the type and number of analyses that can be carried out, depending on the type of the biological material stored. For example, when urine specimens are used as the source of DNA, there will be relatively little DNA available compared to blood samples. However, the use of PCR overcomes some of these restrictions and permits DNA typing in situations that were previously unthinkable. In literature, some studies on DNA typing from urine samples were performed and several variables encountered in extracting DNA from urine samples have been considered (1–7). For example Gasparini *et al.* have reported cystic fibrosis typing using urine samples, while others, e.g., Roest *et al.*, have demonstrated the use of urine samples for population studies. Yokota *et al.* have considered some of the variables encountered in extracting DNA from urine samples. However, in this study the analysis was not carried out on specimens that had been stored at −20°C for a long period.

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0003-2697/02 $35.00

In this study we have examined a subset of urine specimens collected from the DOM (Dutch Diagnostic Study of Breast Cancer) (8) cohort and investigated various parameters which might influence the accuracy of DNA typing, including the total quantity of DNA isolated, the presence of bacterial DNA, the fragmentation of DNA, and the ability to sustain PCR amplification using amplicons of various lengths. Furthermore, we analyzed the observer variation in genotyping a large number of cohort members for a particular marker, namely methylenetetrahydrofolate reductase (MTHFR)[2] polymorphism.

MATERIALS AND METHODS

In the main part of this study we used 69 frozen urine samples from women who had participated in the DOM cohort (8). For the DOM study, overnight urine samples were collected, immediately frozen at intake, and questionnaires were filled in between 1974 and 1986. All the women were asked to consent to the use of their samples in future research, but they were not required to sign an agreement. The Dutch institutional review board for human studies approved our study.

We removed 69 samples from the −20°C storage facility. The urine was thawed overnight at room temperature, mixed vigorously and 50 ml removed for DNA isolation.

DNA Isolation

The alcohol precipitation method was used for 48 DNA isolations as follows. Cells and nuclei were pelleted by centrifugation at 2000*g* for 15 min and then washed twice in phosphate-buffered saline (PBS) (15 min; 2000*g*). DNA was isolated from the pellets as follows: the pellets were resuspended in 500 μl PBS and centrifuged for 5 min at 12,000*g*. Pellets containing cells and nuclei were resuspended in 300 μl cell lysis buffer (10 mM Tris–HCl; 2 mM EDTA; 400 mM NaCl, pH 8.2) and incubated overnight at 55°C. Proteins were precipitated by addition of 100 μl protein precipitation solution of 6 M NaCl and the precipitates pelleted by 5 min centrifugation at 12,000*g*. Thereafter the supernatant was treated with one volume absolute ethanol (room temperature) and centrifuged for 5 min at 12,000*g*. The resulting DNA pellet was washed with 70% ethanol and centrifuged for 5 min at 12,000*g* and finally resuspended in 40 μl 10 mM Tris, 1 mM EDTA, pH 7.6 (TE).

[2] Abbreviations used: DNA, deoxyribonucleic acid; DOM, Diagnostisch Onderzoek Mammacarcinoom (Dutch Diagnostic Study of Breast Cancer); MTHFR, methylenetetrahydrofolate reductase; PCR polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

DNA Isolation with Filtration of Urine Samples

The alcohol precipitation method described above and filtration was used for 21 DNA isolations as follows. The urine samples were split into three portions: the first sample was isolated using normal procedures, for the second portion one filter was used before DNA was isolated, and for the third portion two filters were used. The filtration technique was described by Prinz *et al.* (9). After the first centrifuging, the pellets, after resuspension, were filtered through 40 μm pore size nylon gauze (Millipore), filter 1. After 10 min at 3000*g* the supernatant was discarded and the sediment was resuspended in 1.5 ml PBS and transferred to Eppendorf tubes. The lids of the tubes had been cut off and pierced in the middle leaving only the outer ring. The tubes were now closed by placing a 11 μm pore size nylon gauze (Millipore), filter 2, over the opening and pressing the outer ring of the lid down. The tubes were then inverted, placed in a centrifuge tube on top of a second Eppendorf tube, and centrifuged for 5 min at 1500*g*. The 11-μm mesh retains the human epithelial cells, which can be subsequently removed by opening the tube and applying additional lysis buffer on the top. Two PCRs of different lengths (198 and 547 bp) were used to assess the DNA content in the filtered and nonfiltered samples.

DNA Quantity Determination

The quantity of DNA was measured using PicoGreen (Molecular Probes Europe BV) and an optical density spectrofluorometer (Kotron Analytical, SFM25). The PicoGreen was diluted 1:200 with TE at pH 7.6. Each reaction contained 1 ml of dye solution plus a sample of DNA in 1 ml TE. The solution was mixed and incubated for 2 to 5 min at room temperature. Standard fluorescence curves were constructed by making known serial dilutions of DNA and based on the stoichiometric DNA–PicoGreen binding defined by the commercial provider. PicoGreen-stained samples were excited at 480 nm and the emission intensity was recorded at 520 nm. Each sample was measured twice and the DNA concentrations averaged.

DNA Quality Determination

PCR amplicons from seven different genes, one anonymous polymorphic marker, and a bacterial PCR were generated (MTHFR, VHL, NAT2, HFE, BRCA1, PTEN, HGF, 16SrRNA, D1S1663) using defined primers to yield products of various lengths. Primer sequences and PCR conditions are given in Table 1. The following PCR conditions were used: the MTHFR and VHL PCRs were performed in buffer (67 mM Tris, pH 8.8, 6.7 mM $MgCl_2$ 10 mM 2-mercaptoethanol, 6.7 μM EDTA, pH 8.0, 16.6 mM $(NH_4)_2\ SO_4$), 0.1 mg/ml BSA,

TABLE 1

Characteristics of PCRs

Primer	Locus	Fragment length	Program	Sequence	Orientation
PR 851	MTHFR	198	4 min 94°C; 40 s 94°C, 40 s 55°C, 2 min	5′-aggacggtgcggtgagagtg	Forward
PR 653			72°C; 10 min 72°C	5′-tgaaggagaaggtgtctgcggga	Reverse
PK 1290	HFE	233	10 min 95°C; 30 s 95°C, 50 s 66°C, 30 s	5′-gaaggtgacacatcatcatgtgac	Forward
PK 1291			72°C; 30 min 72°C	5′-ctgggtgctccacctggc	Reverse
PK 465	BRCA1	300	10 min 95°C; 30 s 95°C, 30 s 60°C, 1 min	5′-tcctgacacagcagacattta	Forward
PK 466			72°C; 30 min 72°C	5′-ttggattttcgttctcactta	Reverse
1F355	VHL	327	4 min 94°C; 1 min 94°C, 1 min 55°C, 2 min 72°C	5′-agagtacggccctgaagaagacgg	Forward
1R774				5′-tctggcacgatagcagggac	Reverse
PK 1030	PTEN	398	10 min 95°C; 30 s 95°C, 30 s 55°C, 30 s	5′-gttcatctgcaaaatgga	Forward
PK 1031			72°C; 30 min 72°C	5′-tggtaatctgacacaatgtccta	Reverse
Mix 961020	D1S1663	409–425	10 min 95°C; 30 s 95°C, 30 s 55°C, 1 min 30 s	5′-caacatatgaacaggcggag	Forward
Mix 961020			72°C; 30 min 72°C	5′-agcctcagaaactgagtccc	Reverse
P366-386	NAT2	547	10 min 95°C; 1 min 95°C, 1 min 60°C, 2 min	5′-gctgggtctggaagctcctc	Forward
P891-913			72°C; 10 min 72°C	5′-ttgggtgatacatacacaaggg	Reverse
PK 1303	GF	834	10 min 95°C; 30 s 95°C, 50 s 66°C, 30 s	5′-gccttcccaaccattcccctta	Forward
PK 1304			72°C; 30 min 72°C	5′-gagaaaggcctggaggattc	Reverse
P11P	16rRNA	217	10 min 95°C; 1 min 95°C; 1 min 55°C; 10 s	5′-gaggaaggtggggatgacgt	Forward
P13P			72°C; 10 min 72°C	5′-aggcccgaacgtattcac	Reverse

0.5 pmol of 3′-primer, 0.4 pmol of 5′-primer, 0.125 mM of each nucleotide (Pharmacia Biotech, Uppsala, Sweden), 1.25 U Amplitaq polymerase (Perkin Elmer, U.S.A.), 2 or 5 μl DNA from urine, and 1 μl DNA from blood. The NAT2, HFE, BRCA1, PTEN, D1S1663, and HGF PCRs were performed in Perkin Elmer buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 2.5 mM $MgCl_2$, 0.125 mM of each nucleotide (Pharmacia Biotech), 0.2 mg/ml BSA, 10 pmol of each primer, 1.25 U Amplitaq Gold polymerase (Perkin Elmer). and 2 μl DNA from urine. HFE and HGF PCRs were multiplexed in a single reaction. A PCR was considered successful when a product of the expected length was visible on an agarose gel.

A bacterial-specific PCR for 16S rRNA was performed using the following PCR conditions: buffer (50 mM Tris–HCl (pH 9.0), 50 mM KCl), 7 mM $MgCl_2$, 0.1 mg/ml BSA, 1 pmol of each primer, 0.125 mM of each nucleotide (Pharmacia Biotech), 0.5 U Super Taq (HT Biotechnology, Cambridge, England), and 2 μl DNA from urine. Primer sequences and PCR conditions are given in Table 1.

The genotype determinations of the MTHFR polymorphisms were performed by radioactive dot blot. Dots were visualized and interpreted by eye on the resulting X-ray films. The intra- and interobserver variation for the MTHFR polymorphism (677 C→T) was evaluated in 644 random samples from the DOM cohort. Distinction between the MTHFR 677C and 677T alleles was carried out by hybridization with allele-specific oligonucleotides to PCR products spotted onto membranes as follows: 2.5 μl PCR product from each subject was dotted in duplicate on to hybridization membranes (Hybond N+ nylon transfer membranes, Amersham, Buckinghamshire, UK). Membranes were prepared following the manufacturer's instructions. Both dot blot membranes were prehybridized for 1 h in hybridization mix (6× SSC, 5× Denhardt's, 0.4% SDS, 0.02mg/ml herring sperm DNA (Boehringer Mannheim, Mannheim, Germany)). The MTHFR alleles, 677C and 677T, were detected by hybridizing an antisense oligonucleotide for each allele to a separate membrane overnight: MTHFR 677C (5′-[γ-^{32}P]ATP-CTGCGGAG**C**CGATTTCATC) and MTHFR 677T (5′[γ-^{32}P]ATP-TGCGGGAG**T**CGATTTCAT). The membranes were washed twice for 10 min in 2× SSC, 0.1% SDS at room temperature. Nonspecific binding was removed by 30 min of washing in 2× SSC, 0.1% SDS at 57°C for the 677C hybridization and at 58°C for the 677T hybridization. For genotype determination both X-ray films are read at the same time. The presence of a dot on the 677C membrane revealed a homozygote 677C person, the presence of a dot on the 677T membrane indicated a homozygote 677T women, and the presence of a dot on both membranes revealed a 677T/677C heterozygote individual. The samples were classified by one observer twice and by two different observers once. In the intraobserver tests samples were excluded for which one observer was unable to score a person at least once. For the interobserver variation those samples that were not genotyped by at least one observer were excluded. These exclusions were amplified and dot blotted again to evaluate the improvement. Kappa's were computed as measures that adjust the percentages of

TABLE 2

Quantity and Quality of DNA, Isolated from Frozen Urine Samples

DNA amount isolated from 50 ml urine	PCR 200-bp range				PCR 300-bp range				PCR 400-bp range				PCR 800 bp			
	MTHFR 198 bp		HFE 233 bp		BRCA1 300 bp		VHL 327 bp		PTEN 398 bp		D1S1663 425 bp		HGF 834 bp		Total	
0–0.1 μg	7/9	77%	3/7	43%	2/5	40%	4/9	44%	2/5	40%	1/9	11%	3/7	43%	22/51	43%
0.1–20 μg	36/39	92%	16/23	70%	12/21	57%	34/39	87%	8/17	47%	11/39	28%	8/23	35%	125/202	62%
Total	42/48	88%	19/30	63%	14/26	54%	38/48	79%	10/22	45%	12/48	25%	11/30	36%	147/253	58%

Note. Samples are divided into two categories according to the amount of DNA isolated from 50 ml of frozen defrosted urine, and several PCRs of different and equal lengths were performed. Absolute amount and percentage of visible PCR products on agarose gel are shown.

agreement for chance, and 95% confidence intervals were calculated (10).

RESULTS

The quantities of DNA isolated from 48 samples of 50 ml frozen urine samples ranged from 0 ng to 20 μg, the 25th percentile was 4 ng/μl, median was 14 ng/μl, and 75th percentile was 28 ng/μl. Fifteen samples exhibited visible, high-molecular-weight DNA on agarose gels following ethidium bromide staining. All visible samples had a DNA concentration of 13 ng/μl or more.

A filtration step before isolating DNA was performed in 21 urine samples to optimize the quality of the DNA. After filtration and isolation, a MTHFR (198 bp) and NAT2 (547 bp) PCR was performed in these samples. This revealed no significant difference between the filtered and unfiltered specimens for the two reactions. In another series involving 10 urine samples, we assessed the level of bacterial contamination by microscopic observation of the samples and a bacterial specific PCR for 16S rRNA was performed. There were large differences observed in the bacterial contamination, varying from 0 to high, and there was no apparent correlation with the success of amplification for the seven human PCRs tested.

During the PCR experiments in another subset of urine samples, the amount of DNA available for some samples was insufficient to carry out the intended number of PCRs for each of the 48 samples. Only a single correctly sized PCR band was obtained in all cases scored as positive. In the case of the shortest fragment (198 bp) 42 of 48 (88%) revealed a product visible on agarose gel. As shown in Table 2, there was a general trend toward lower success percentage with increasing amplicon length, although this was not absolute. For example, VHL, with a length of 327 bp, scored higher at 79% than the BRCA1 (300 bp) with 54%. The D1S1663 (425 bp) PCR amplicon scored much lower than the HGF (834 bp). This suggests that not only amplicon length determines the success frequency but that the specificity of the primers and amplification conditions may also be important. Furthermore, the DNA concentration of template DNA appeared to have a greater negative effect on PCRs with longer amplicons. However, since the number of PCRs performed was sometimes small due to lack of sufficient DNA, these percentages and trends must be interpreted with caution.

In the genotyping experiment, 644 women were classified as MTHFR homozygote 677 CC, heterozygote 677 CT, homozygote 677 TT, or unknown. After repeat classification by the same observer, it was possible to score the alleles in 501 of 644 (77.8%) samples. After excluding unknowns, the κ for intraobserver agreement was 0.86 (95% confidence interval 0.82, 0.90) (Table 3). The two different observers did not agree on the genotype determination or determined both unknown in 137 individuals (21.3%). The κ for interobserver agreement was 0.88 (95% confidence interval 0.84, 0.92) (Table 4). Samples of the 137 women were amplified, dot blotted, and scored again. From those samples 10 were determined as homozygote 677 CC, 42 were heterozygote 677 CT, and 4 revealed homozygote 677 TT. The two observers did not agree on the genotype determination in 20 individuals or determined both unknown in 61 women. After discussion this ultimately resulted in a success rate of 89.3% and the genotype frequencies are shown in Table 5.

DISCUSSION

Urine samples can be used as a source of DNA for determining genotypes. This study reveals some additional considerations if urine is used for genotyping. The amount of DNA is low and was therefore not generally detectable either by ethidium bromide staining on a gel or by standard UV absorption spectroscopy. A third (33%) of the samples were visible on agarose gel and showed high-molecular-weight DNA. We noted that the PicoGreen fluorescent detection gave the most reliable results with such low DNA concentrations. Prinz *et al.* (9) reported that the amount of total human DNA that could be extracted

TABLE 3

Intraobserver Agreement in MTHFR Genotyping in DNA, Isolated from 644 Frozen Urine Samples

	Observer A second time				
	Homozygote	Heterozygote	Homozygote	Unknown	Total
	677 CC	677 CT	677 TT		
Observer A first time					
Homozygote 677 CC	220	13	0	3	236
Heterozygote 677 CT	22	222	5	12	261
Homozygote 677 TT	3	2	59	1	65
Unknown	6	4	5	67	82
Total	251	241	69	83	644

Note. Individuals were determined as homozygote wild type, heterozygote, or homozygote mutant.

from 20 ml of fresh urine ranged from 20 to 40 ng for males and 400 to 800 ng for females (female urine contains more epithelial cells). In this study, we were able to isolate the same amount of DNA (400–500 ng) from women's frozen 50-ml urine samples in only 50% of the samples. This is possibly due to storage conditions, temperature, and time (20°C in our study vs 4°C in Prinz *et al.* (9) and 16 years in our study vs. 1–7 years in Prinz *et al.* (9)). However, their results demonstrated that quantity of DNA can be a limiting factor and suggested that male urine specimens, stored for a long period, may be of very limited use.

PCR amplification for various primers and amplicon lengths revealed a general tendency for a negative correlation between amplicon length and success rate of the reaction. There were unexpected differences between specimens, which could possibly be explained by the diversity of the samples and efficiency of the primers to bind to template DNA. In addition, there were obvious differences between samples in the degree of bacterial contamination and the presence of crystal deposits, which may influence the priming efficiency.

Prinz *et al.* (9) proposed that removal of bacteria from urine preparation improves the success rate of PCR amplification. In that study of the total number of samples 25% proved to be contaminated and removal of fungi and bacteria was effective. In our study we did not select contaminated samples, and maybe therefore we could not observe a significant difference between the unfiltered and filtered specimens for the MTHFR (198 bp) and NAT2 (547 bp) reactions. In a second series involving 10 specimens, we observed large differences in the bacterial contamination, but there was no apparent correlation with the success of amplification for the seven human PCRs tested. Accordingly, we conclude that the extra time spent removing bacteria prior to DNA isolation is not necessary.

The ±425-bp amplicon of an anonymous CA repeat polymorphic marker D1S1663 amplified poorly over the full range of specimen DNA concentrations. In concentrations of more than 100 ng the reaction did not occur in 62% of the cases. Therefore, our conclusion is that, while there may be a general tendency for successful amplification with short amplicons and with higher concentrations of DNA templates, there are exceptions to this. Consequently, in practice, each PCR marker has to be tested on various sources of urine DNA to determine its success rate before carrying out

TABLE 4

Interobserver Variation in MTHFR Genotyping in DNA, Isolated from 644 Frozen Urine Samples

	Observer B				
	Homozygote	Heterozygote	Homozygote	Unknown	Total
	677 CC	677 CT	677 TT		
Observer A					
Homozygote 677 CC	236	9	1	5	251
Heterozygote 677 CT	23	213	1	4	241
Homozygote 677 TT	2	2	58	7	69
Unknown	15	1	1	66	83
Total	276	225	61	82	644

Note. Individuals were determined as homozygote wild type, heterozygote, or homozygote mutant.

TABLE 5

MTHFR Genotype Frequencies in 644 Controls Using Two Different Observers and Repeat Amplification and Classification of Inconsistent Outcomes

Genotype MTHFR	*n*	%
Homozygote 677 CC	246	38.2
Heterozygote 677 CT	262	40.6
Homozygote 677 TT	67	10.4
Unknown by one observer	8	1.2
Unknown by both observers	61	9.5
Total	644	100

large-scale studies. Although in this study we have measured DNA to permit us to compare concentrations between various specimens, it seems that the results do not critically depend on concentration. In practice it would take much time to measure concentrations in large studies and in the process of carrying out PCRs, those specimens that consistently fail to give a PCR product will be evident. It is probably more efficient to simply remove such samples from the observation. Although in this study we have used standard primer pairs available in the literature it may be necessary to define other primers and priming conditions to maximize the amplification success rate in urine samples.

We have compared the inter- and intraobserver reproducibility for the genotyping of the MTHFR marker, which behaved more consistently over all DNA concentrations and gave a much higher frequency of amplification success than the other six markers tested. It was noted that the inconsistencies for both inter- and intraobserver observations occurred primarily in the typing of homozygote CC and heterozygote CT. In contrast, the distinction between heterozygote CT and homozygote TT generated less ambiguity. In the future, other methods may be used on urine samples, based on Taqman or molecular beacon assays. These are both sensitive and specific and are easier to automate, but have the significant disadvantage of still being extremely expensive.

Although the κ's for observational agreement are high, values may be lower than would be expected from blood. Moreover, in nested case control studies when cases and controls are analyzed blind, these misclassification errors will be nondifferential. A comparison of blood and urine samples from the same individuals makes it possible to investigate this aspect.

Despite the difficulties presented in this article, it should be emphasized that the epidemiological value of a cohort such as the DOM is enormous in terms of the availability of information on relatively common diseases. The technical disadvantages of using DNA isolated from urine are far outweighed by the information gained. We were surprised that so much genotype information can be gathered from such an unexpected source as urine. However, the quantities of DNA that can be isolated are limited and other sources of DNA should be used wherever possible.

Urine collection can therefore be considered as a useful method of obtaining DNA in large cohort studies and other circumstances when blood samples cannot be obtained or have not been stored.

ACKNOWLEDGMENTS

We thank D. L. van de A, V. Veenbergen, and B. Slotboom for assisting in identifying the frozen samples, isolating and amplifying DNA, and classifying the X-ray films. We also thank F. de Waard and H. J. A. Collette for their perseverance and work in collecting the urine samples in the initial phase of the cohort study. Financial support for this study was obtained from the Dutch Cancer Society (UU 98-1707).

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