Quantifying cell-free DNA in urine: comparison between commercial kits, impact of gender and inter-individual variation

Greta Streleckiene[1,2], Hayley M Reid[2], Norbert Arnold[2,3], Dirk Bauerschlag[3] & Michael Forster*[,2]
[1]Institute for Digestive Research, Lithuanian University of Health Sciences, Eiveniu Str. 2, LT-50009 Kaunas, Lithuania, [2]Institute of Clinical Molecular Biology, Christian-Albrechts-Universität zu Kiel, Schleswig-Holstein, D-24105 Kiel, Germany, [3]Department of Gynecology & Obstetrics, University Hospital of Schleswig-Holstein, Christian-Albrechts-Universität zu Kiel, D-24105 Kiel, Germany

BioTechniques 64: 225-230 (May 2018) 10.2144/btn-2018-0003
Keywords: circulating cell-free DNA • DNA amount • urine

DNA can enter the blood circulation from living cells by extracellular vesicles or at cell death, and pass into urine through the kidney barrier. Urine can be collected non-invasively, making it an interesting source of cell-free DNA (cfDNA) for research studies and ultimately for clinical diagnostics. However, there is currently a lack of data on the quantity and variability of cfDNA in urine. Here, we benchmark two commercial urine cfDNA isolation kits with respect to the quantity of DNA, the labor time, and cost. The results show distinctive differences between each kit. Furthermore, the cfDNA amount from the same probands varied strongly from day to day and may be higher in female samples than in male samples ($p = 0.003$).

Cell-free DNA is released from cells at cell death and also through active cell secretion [1–3]. cfDNA is readily available in various body fluids, such as blood plasma and urine [4–6]. One advantage of urine over blood is non-invasive sampling at the study probands' or patients' own choice of time, without the need for medical staff. Urine sampling could improve proband recruitment success and compliance, and avoids potential ethical problems in studies that involve children or patients in great pain. In contrast to blood cfDNA, there are insufficient data on the quantity and variability of cfDNA in urine. This fundamental information is required for designing urine cfDNA studies.

Here, we benchmarked two new commercial urine cfDNA isolation kits from Norgen and PerkinElmer with respect to cfDNA yields, replicability of yields from the same probands' urine at sequential intervals, and potential differences between males and females. We chose these specific urine kits because the Norgen blood plasma cfDNA isolation kits showed the highest yields in a benchmark [7] and the Perkin-Elmer plasma cfDNA kit yields in our lab were even higher (data not shown).

Informed consent was obtained from all participants, consisting of healthy volunteers and cancer patients. The University Hospital of Schleswig-Holstein's ethics committee approved the patient information sheet and the consent form used for the study (B327/10, D470/14). All patients included in the study gave written informed consent to donate their samples to the biobank for research use. The research was conducted according to the principles of the Declaration of Helsinki. Initially, we collected samples from cancer patients and healthy volunteers in Urine Collection and Preservation Tubes (15 cc) (Norgen Biotek, Cat. 18120) and isolated cfDNA using the Perkin-Elmer kit and the Norgen kit. The cfDNA yields ranged from 0 to 86.5 ng cfDNA from 5 ml of urine (data not shown). It was unclear whether this variability was of biological nature.

Therefore, we systematically compared the kits by examining the reproducibility of urine cfDNA yield from eight healthy volunteers (four females and four males) on 5 different days. They were informed that the second morning urine was required, as the first morning urine was reported to contain more degraded cfDNA [8]. 100 ml sterile disposible urine cups were distributed. Urine donation took place between 9am and 10am on each day of donation. No preservation agents were used. Urine processing started between 10am and 11am on the same day. The urine samples were centrifuged twice to remove cellular matter, first at 200 x g (10 min) followed by 1800

METHOD SUMMARY

Two new kits were benchmarked for urine cfDNA isolation: magnetic bead based isolation (NEXTprep-Mag Urine cfDNA Isolation Kit, Cat. NOVA-3826–02, PerkinElmer, MA, USA) and silica gel membrane column-based isolation (Urine Cell-Free Circulating DNA Purification Midi Kit, Cat. 56700, Norgen Biotek, ON, Canada). The quantity and length distribution of cfDNA were evaluated by an automated electrophoresis system (Agilent 2200 TapeStation using the High Sensitivity D5000 Screen Tape, Cat. 5067–5588, Agilent Technologies, Waldbronn, Germany).

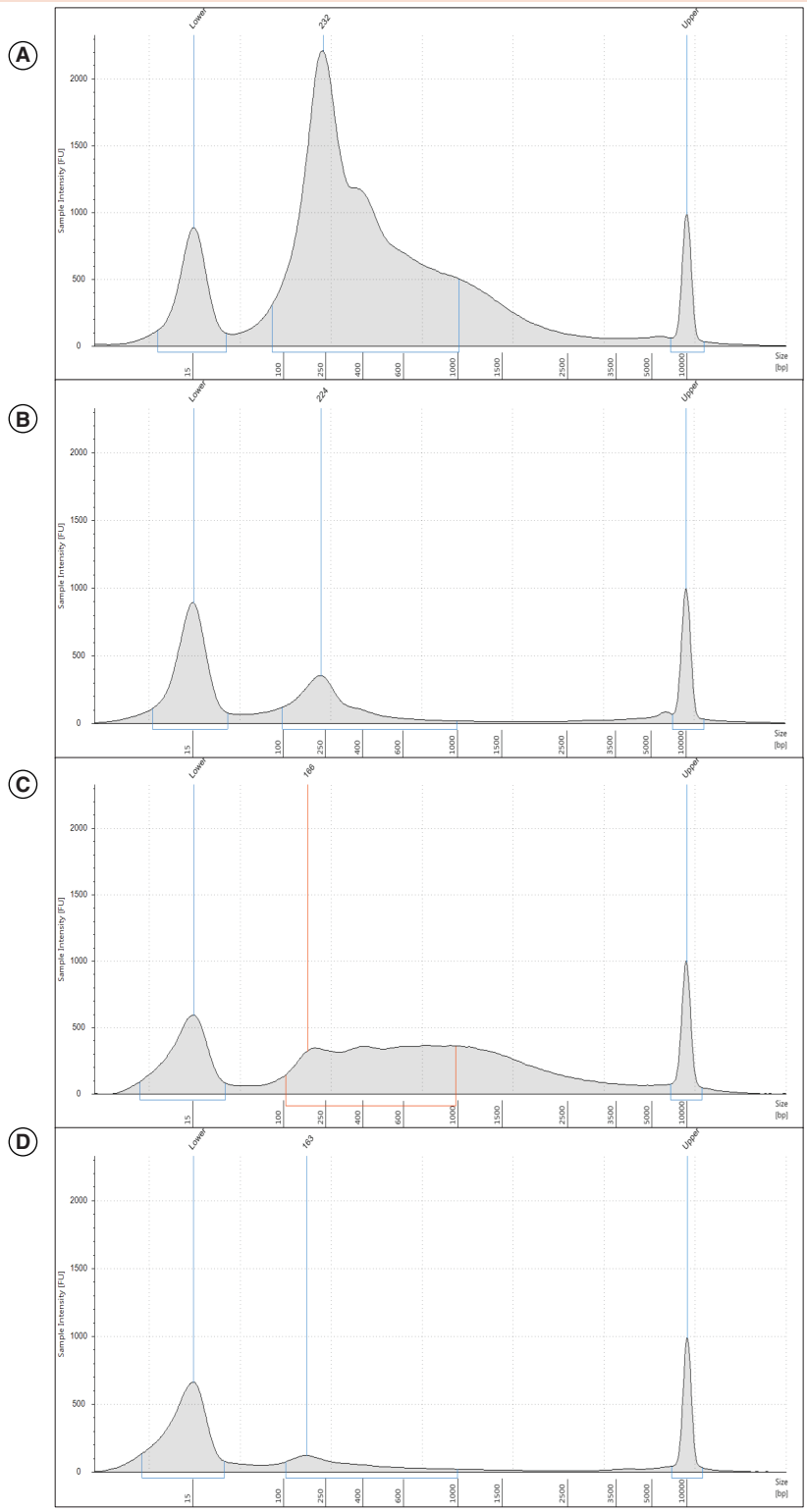

Figure 1. Urine cfDNA length distributions depending on gender and kit. The figure shows Agilent TapeStation electropherograms of cfDNA from pooled urine samples from the fourth donation day. **(A)** Isolated with PerkinElmer kit from pooled healthy female urine. **(B)** Isolated with PerkinElmer kit from pooled healthy male urine. **(C)** Isolated with Norgen kit from same pooled female urine. **(D)** Isolated with Norgen Kit from same pooled male urine. The DNA fragment size range from 100–1000 base pairs was manually selected for the quantification of the cfDNA yields.

x g (10 min). Equal volumes from the four female or four male samples were pooled to obtain an average result for female or male donors without the cost of isolating each individual's cfDNA. 10 ml of each pool was used for Norgen-based cfDNA isolation and 4 ml for PerkinElmer-based isolation. Additionally, on the fifth day of donation, cfDNA was isolated from each individual's urine sample (F1–F4 and M1–M4). All isolations were performed according to manufacturers' instructions. Length distribution and concentration of isolated DNA were analyzed using the Agilent TapeStation, according to the manufacturer's instructions. A DNA size range of 100 to 1000 bp was manually selected in the TapeStation Analysis Software A.01.04 to obtain the cfDNA concentration.

Figure 1 compares electropherograms between isolates from the same pooled female and male urine samples, respectively. Figure 2A and Table 1 summarize the cfDNA yield ranges and differences between kits in the female and male pools on five donation days. The mean yields of cfDNA (ng per ml of urine ± standard deviation) were 4.72 ± 3.53 (Norgen) and 14.83 ± 11.16 (PerkinElmer) for female pools and 0.83 ± 0.39 (Norgen) and 2.04 ± 0.33 (PerkinElmer) for male pools. The yield difference between kits was significant for male pools ($p = 0.008$, Wilcoxon test). The yield difference between genders was significant for the Norgen kit ($p = 0.016$, Wilcoxon test) and when both kits were considered ($p = 0.003$, Wilcoxon test). Figure 2B and Table 2 indicate yield ranges and kit-based differences for the eight probands' individual urine samples that were isolated on the fifth day. The mean yields of cfDNA (ng per ml of urine ± standard deviation) were 0.98 ± 0.40 (Norgen) and 2.27 ± 1.17 (PerkinElmer) for female individuals and 1.46 ± 0.67 (Norgen) and 1.99 ± 1.02 (PerkinElmer) for male individuals. Finally, Table 3 summarizes the isolation cost and processing times.

In conclusion, the bead-based method was twice as fast as the column-based method and tended to yield more cfDNA per ml of urine. Larger sample numbers may lead to greater clarity. The urine cfDNA length profiles (Figure 1) suggest that the PerkinElmer kit is more efficient at capturing short DNA. Short DNA is of scientific interest, as it is present in blood plasma as cfDNA. In blood plasma, cfDNA lengths peak at about 165 nucleotides with a minor peak at about 1000 nucleotides [9]. In urine, we observed prominent DNA fractions longer than 165 nucleotides. These fractions of longer DNA fragments possibly arise from the epithelia of the urinary tract, after shedding

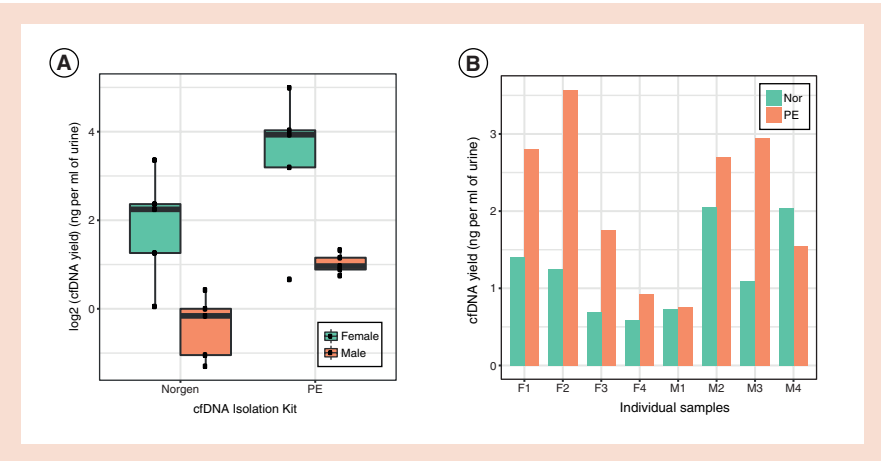

Figure 2. Urine cfDNA isolation yields depending on kit and individual. (A) Pooled urine isolations. Norgen and PerkinElmer cfDNA isolation yields from the same healthy female and male urine pools replicated on five days. Yield varies strongly within the same groups of study probands between different days. Yield from the same urine is generally higher with the PerkinElmer kit. Yield is higher in the female pools. **(B)** Individuals' urine isolations. Norgen and PerkinElmer cfDNA isolation yields from four healthy female probands' individual urine samples (F1-F4) and from four healthy male probands' individual urine samples (M1–M4). The PerkinElmer kit yields are higher than the Norgen kit yields, especially for the female urine. Fourfold inter-individual differences are seen between lowest and highest yield.

and subsequent lysis of epithelial cells. Our gender-related differences in cfDNA yields correspond to a previous study on genomic DNA in urine [10,11]. Higher DNA yields in female urine have been previously reported [11–13] indicating that urine from females contained more epithelial cells than that from males.

To sum up, there is vast variation in DNA yields between different individuals and even for the same individuals on different days. When designing a study, we recommend ample amounts of urine to be collected. For NGS with 50 ng of cfDNA, we recommend collecting 60 ml urine for the PerkinElmer kit (15 extractions × 4 ml) or 70 ml for Norgen (7 extractions × 10 ml using the Midi kit or 3 × 30 ml using the Maxi kit).

Author contributions

GS, HR and MF prepared the manuscript. GS and HR performed cfDNA isolations, cfDNA library preparation and sequencing experiments. GS and MF analyzed the data. NA and DB provided cancer patient samples and helped write the manuscript. All authors read and approved the final version.

Acknowledgements

We are grateful to the urine donors who participated in this study. Regina Fredrik significantly helped with the lab work. Andre Franke, Juozas Kupcinskas and Philip Rosenstiel provided lab facilities. Andre Franke edited the manuscript.

Ethical conduct of research

Informed consent was obtained from all participants, consisting of healthy volunteers and cancer patients. The University Hospital of Schleswig-Holstein's ethics committee approved the patient information sheet and the consent form used for the study (B327/10, D470/14). All patients included in the study gave written informed consent

Table 1. cfDNA yields from female and male urine sample pools† on five different collection days.

Donation day	Isolation kit	Gender of pool	cfDNA (ng per ml of urine)	cfDNA per urine isolation (ng)‡
1.	Norgen	F	4.75	47.45
		M	1.00	10.00
	PerkinElmer	F	15.25	61.00
		M	1.68	6.72
2.	Norgen	F	10.25	102.50
		M	0.48	4.85
	PerkinElmer	F	31.80	127.20
		M	2.23	8.90
3.	Norgen	F	5.15	51.50
		M	0.90	8.95
	PerkinElmer	F	16.35	65.40
		M	1.85	7.40
4.	Norgen	F	2.40	23.95
		M	0.41	4.07
	PerkinElmer	F	9.15	36.60
		M	2.51	10.04
5.	Norgen	F	1.04	10.37
		M	1.34	13.42
	PerkinElmer	F	1.58	6.34
		M	1.95	7.81

†Female urine pools from the same four healthy females, male urine pools from the same four healthy males.
‡Urine volumes in the cfDNA isolation protocols are 10 ml (Norgen) vs 4 ml (Perkin Elmer).

Table 2. cfDNA isolation results from eight healthy individuals' urine samples.

Gender	Sample	Isolation kit	cfDNA (ng per ml of urine)	cfDNA per urine sample (ng)†
Female	F1	Norgen	1.40	14.03
		PerkinElmer	2.81	11.22
	F2	Norgen	1.25	12.49
		PerkinElmer	3.58	14.30
	F3	Norgen	0.69	6.90
		PerkinElmer	1.75	7.01
	F4	Norgen	0.59	5.94
		PerkinElmer	0.92	3.70
Male	**M1**	**Norgen**	**0.73**	**7.32**
		PerkinElmer	**0.76**	**3.03**
	M2	**Norgen**	**2.05**	**20.54**
		PerkinElmer	**2.70**	**10.79**
	M3	**Norgen**	**1.09**	**10.89**
		PerkinElmer	**2.94**	**11.77**
	M4	**Norgen**	**2.04**	**20.35**
		PerkinElmer	**1.55**	**6.20**

†Urine volumes in the cfDNA isolation protocols are 10 ml (Norgen) vs 4 ml (Perkin Elmer).

Table 3. Commercial cfDNA isolation kits used in this study.

Full name of kit	Manufacturer	Urine amount (ml)	Elution volume (μl)	Price per sample (€)	Processing time (min)
Urine Cell-Free Circulating DNA Purification Midi Kit	Norgen Biotek	10	50	20.50	90
NextPrep-Mag Urine cfDNA Isolation Kit	Bioo Scientific by Perkin Elmer	4	20	12.17	45

to donate their samples to the biobank for research use. The research was conducted according to the principles of the Declaration of Helsinki.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

Open access

References

1. Thierry AR, El Messaoudi S, Gahan PB, Anker P, Stroun M. Origins, structures, and functions of circulating DNA in oncology. *Cancer Metastasis Rev.* 35(3), 347–376 (2016).
2. Thakur BK, Zhang H, Becke A Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res.* 24(6), 766–769 (2014).
3. Minciacchi VR, Zijlstra A, Rubin MA, Di Vizio D. Extracellular vesicles for liquid biopsy in prostate cancer: where are we and where are we headed? *Prostate Cancer Prostatic Dis.* 20(3), 251–258 (2017).
4. Botezatu I, Serdyuk O, Potapova G Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. *Clin. Chem.* 46(8 Pt 1), 1078–1084 (2000).
5. Chan KCA, Leung SF, Yeung SW, Chan ATC, Lo YMD. Quantitative analysis of the transrenal excretion of circulating EBV DNA in nasopharyngeal carcinoma patients. *Clin. Cancer Res.* 14(15), 4809–4813 (2008).
6. Birkenkamp-Demtröder K, Nordentoft I, Christensen E Genomic alterations in liquid biopsies from patients with bladder cancer. *Eur. Urol.* 70(1), 75–82 (2016).
7. Mauger F, Dulary C, Daviaud C, Deleuze JF, Tost J. Comprehensive evaluation of methods to isolate, quantify, and characterize circulating cell-free DNA from small volumes of plasma. *Anal. Bioanal. Chem.* 407, 6873–6878 (2015).
8. Brisuda A, Pazourkova E, Soukup V Urinary cell-free DNA quantification as non-invasive biomarker in patients with bladder cancer. *Urol. Int.* 96(1), 25–31 (2016).
9. Jiang P, Lo YMD. The long and short of circulating cell-free DNA and the ins and outs of molecular diagnostics. *Trends Genet.* 32(6), 360–371 (2016).
10. El Bali L, Diman A, Bernard A, Roosens NHC, De Keersmaecker SCJ. Comparative study of seven commercial kits for human dna extraction from urine samples suitable for DNA biomarker-based public health studies. *J. Biomol. Tech.* 25(4), 96–110 (2014).
11. Chiarella P, Carbonari D, Capone P Susceptibility biomarker detection in urine exfoliate DNA. *Biomark. Med.* 11(11), 957–966 (2017).
12. Vu NT, Chaturvedi AK, Canfield DV. Genotyping for DQA1 and PM loci in urine using PCR-based amplification: effects of sample volume, storage temperature, preservatives, and aging on DNA extraction and typing. *Forensic Sci. Int.* 102(1), 23–34 (1999).
13. Nakazono T, Kashimura S, Hayashiba Y, Hara K, Miyoshi A. Successful DNA typing of urine stains using a DNA purification kit following dialfiltration. *J. Forensic Sci.* 50(4), 860–864 (2005).

Received 30 January 2018; Accepted for publication: 12 April 2018

Address correspondence to: Michael Forster, Institute of Clinical Molecular Biology, Christian-Albrechts-Universität zu Kiel, Schleswig-Holstein, D-24105 Kiel, Germany. E-mail: m.forster@ikmb.uni-kiel.de