Stabilization of cfDNA and Blood Cells in Urine Using a Preservative Reagent and its Compatibility with Urine Analyzers

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ABSTRACT

Background: Circulating cell-free DNA (cfDNA) in blood plasma derived from tumor, fetus and transplanted organs has been extensively studied. These circulating cfDNA can pass from the blood through the kidney barrier into urine. The obvious advantage of urine sampling makes urine a useful source of fetal and tumor DNA for the development of noninvasive prenatal and cancer diagnostic and prognostic tests. However, the inherent instability of cfDNA in urine hinders the clinical utility of cfDNA in urine. Nucleated cells in urine could also release genomic DNA into urine leading to an increased DNA background during sample processing and storage. This study was designed to demonstrate an innovative, customer friendly and cost-effective preservative reagent that can preserve cfDNA and blood cells in urine post specimen collection, with minimal effects on urinalysis.

Methods: The effect of different Streck Cell-Free DNA Urine Preserve concentrations on stability of urinary cfDNA was examined and compared to untreated urine samples. The urine samples were diluted 5:1 or 20:1 with the preservative reagent and stored at room temperature with untreated urine specimens in parallel. Aliquots were removed at specified time points. cfDNA was purified from urine and quantified by a Droplet Digital PCR (ddPCR) assay. To study the urine blood cell stability, blood buffy coat was collected and spiked into urine samples with or without the preservative reagent. Blood cells were counted on an automated urine sediment analysis system over 7 days at room temperature. The compatibility of the reagent with urine analyzers was examined by comparing performance of preserved and unpreserved urine on both sediment and chemistry analyzers such as Sysmex UF-1000i™ and CLINITEK Atlas®.

Results: Untreated urine samples showed a significant decrease in cfDNA concentrations of both oncogene KRAS and housekeeping gene β-actin on day 4 and 7 post specimen collection at room temperature. In contrast, cfDNA concentrations remained stable for at least 7 days in urine samples treated 5:1 to 20:1 with Streck Cell-Free DNA Urine Preserve. The preservative reagent also stabilized blood cells in urine at room temperature for up to 7 days, whereas untreated samples showed a significant degradation of urine blood cells. Moreover, the reagent showed a minimal effect on automated urine analysis.

Conclusion: Our results show that Streck Cell-Free DNA Urine Preserve is capable of preserving circulating cfDNA and blood cells in urine. Urine samples preserved with the reagent are generally compatible with urine analyzers. The novel urine preservative reagent can provide a method for obtaining high quality stabilized urinary cfDNA for clinical diagnostics development and application. This allows great flexibility and convenience in sample processing, handling, shipping and storage for both cfDNA and routine urinalysis.

INTRODUCTION

The discovery of cell-free nucleic acids in the circulation has opened up new opportunities for non-invasive diagnostic applications in cancer testing and prenatal diagnosis[1]. Since the confirmation of cfDNA presence in urine[2], there has been much interest in the potential utility of urinary DNA for clinical diagnostic development. The main advantage of urine over other body fluids (e.g., blood) is that urine sampling is truly non-invasive and it can be obtained safely and in large amounts with very limited training. When employing urinary cfDNA, however, it is important to minimize release of cellular DNA from nucleated cells and stabilize cfDNA following urine collection since cfDNA targets are present at low quantities and degrade rapidly[3]. Therefore, it is necessary to address pre-analytical issues that arise during the time between urine collection and cfDNA isolation. These include delay in urine processing and specimen storage temperature. Such conditions may cause cellular DNA contamination and subsequently alter cfDNA levels circulating in urine. Thus, in order to obtain reproducible results, it is essential to standardize the pre-analytical procedure for urine sample handling. cfDNA preservation and stabilization in urine should be an integral part of the non-invasive diagnostic test development using urine as the source of genetic material. In this study we introduced a novel and easy-to-use preservative reagent that can maintain the cfDNA concentration in urine post specimen collection. Stabilization of blood cells in urine was also assessed using the preservative reagent. In addition, the compatibility of preserved urine samples with automated urine analyzers was examined, as automated urinalysis is being widely used to improve the workflow of routine urine analysis.

MATERIALS AND METHODS

- **Urine Collection**

  The morning urine collected from healthy volunteers was treated (5:1 or 20:1) with Streck Urine Preservative and stored at room temperature with untreated samples in parallel. Urine samples were run on urine analyzers or processed for cfDNA testing.

- **Sample Processing for cfDNA testing**

  Aliquots of urine (5 mL) were removed from each sample on day 0, 4 and 7, respectively. These aliquots were centrifuged at room temperature at 4000 rpm (2680 x g) for 10 minutes. 4 mL of supernatant was carefully removed without disturbing pellets and transferred to a new tube using a pipette, followed by cfDNA extraction.

- **Urine cfDNA Isolation**

  Urine cfDNA was purified using the commercially available QIAamp® Circulating Nucleic Acid Kit (QIAGEN®, Santa Clarita, CA). For optimal results, the manufacturer's recommended protocol was modified slightly by increasing the duration of the proteinase K treatment from 30 min to 1 hour at 60 °C.

- **Droplet Digital PCR (ddPCR)**

  Absolute quantification of cfDNA by PCR was performed using the QX100 Droplet Digital PCR system (Bio-Rad, Hercules, CA). The KRAS copy number assay kit was purchased from Applied Biosystems (Foster City, CA). Primers and the probe for the ddPCR quantification of human β-actin were purchased from Integrated DNA Technologies (Coralville, IA).

RESULTS

Figure 1A: 5:1

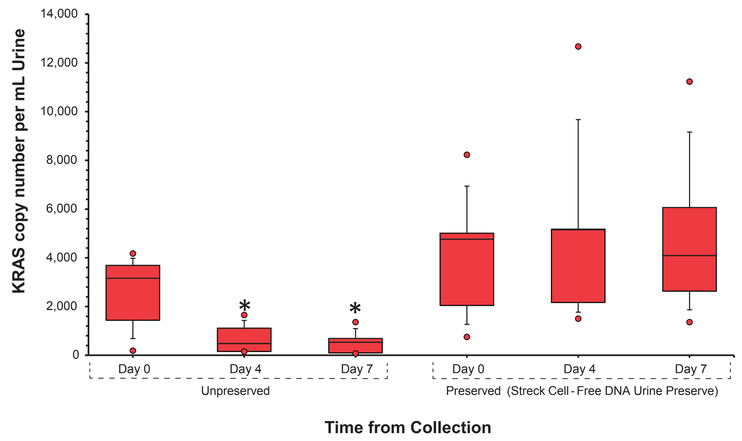

Figure 1B: 20:1

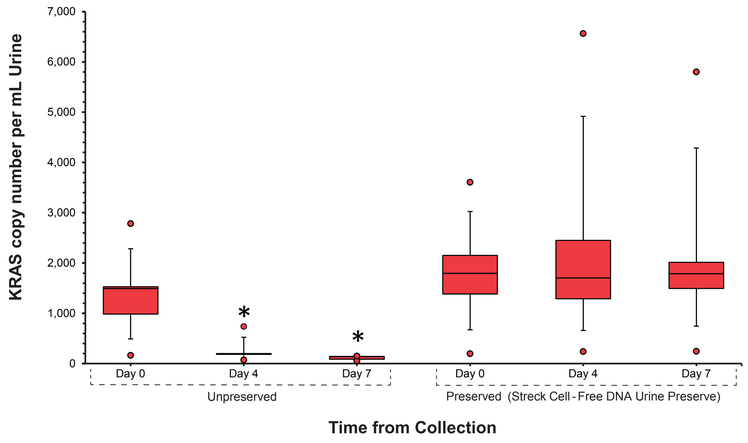

Figure 1. Urinary cfDNA was stabilized by Streck Cell-Free DNA Urine Preserve over a wide range of dilution ratios at room temperature. Urine was treated 5:1 (A) or 20:1 (B) with Streck Cell-Free DNA Urine Preserve and stored at room temperature. On day 0, 4 and 7, cfDNA was isolated from urine samples and quantified by ddPCR. The concentration of KRAS gene decreased significantly in untreated urine on day 4 and 7, whereas it remained stable in all treated urine samples for at least 7 days post specimen collection. Similar results were observed for the house-keeping gene β-actin (data not shown).

Box plots show the median (line inside the box) and 75th and 25th percentiles (limits of the box). The upper and lower error bars indicate the 90th and 10th percentiles, respectively. The upper most and lower most dots indicate the maximum and minimum values. (n=5, *p <0.05)

Table 1

		Unpreserved Control		Preserved (urine:reagent = 20:1)	
		RBC	WBC	RBC	WBC
Low Cell Level	Day 0	682	8.7	637	10.0
	Day 3	314	4.7	633	10.0
	Day 7	25	3.7	611	8.6
Results		Unstable	Unstable	Stable	Stable
High Cell Level	Day 0	6961	92.9	6646	88.4
	Day 3	5594	62.0	6473	87.1
	Day 7	1936	30.1	6315	85.6
Results		Unstable	Unstable	Stable	Stable

Table 1. Blood cells in urine were stabilized by Streck Cell-Free DNA Urine Preserve at room temperature. Buffy coat cells were isolated from whole blood. Blood cells in the buffy coat were counted and spiked into urine samples at two different levels with or without the preservative reagent (urine 20:1 reagent). Spiked blood cells in urine were counted on an automated urine sediment analysis system (i.e., Sysmex UF-1000i™) on day 3 and 7 at room temperature. The preservative reagent stabilized blood cells in urine at room temperature for up to 7 days, whereas untreated samples showed a significant decrease in urine blood cell counts over time.

Table 2

UF-1000i™		Unpreserved	Preserved
Donor #1 female	RBC	1.3	0.95
	WBC	5.3	3.89
	EC	2.6	2.00
	Cast	0.48	0.25
	BACT	0.7	1.05
	*Cond.	12.6	15.5
	X'TAL	0	0.2
Donor #2 female	RBC	16.4	15.7
	WBC	5.9	7.6
	EC	12.8	16.8
	Cast	0.24	0.4
	BACT	196.5	167.5
	*Cond.	24.8	26.5
	X'TAL	0.3	0.1
Donor #3 male	RBC	9.2	6.6
	WBC	2.9	2.6
	EC	2.1	2.1
	Cast	0.12	0.0
	BACT	45.3	32.0
	*Cond.	13.6	15.7
	X'TAL	0.1	0.1

CLINITEK Atlas®		Unpreserved	Preserved
Donor #1 female	COL	yellow	yellow
	CLA	clear	clear
	GLU	neg	neg
	BIL	neg	neg
	KET	neg	neg
	*SG	1.010	1.015
	BLO	neg	neg
	*pH	5.5	6.0
	PRO	neg	neg
	URO	0.2	0.2
	NIT	neg	neg
	LEU	neg	neg
Donor #2 female	COL	yellow	yellow
	CLA	clear	clear
	GLU	neg	neg
	BIL	neg	neg
	KET	neg	neg
	*SG	1.020	>=1.030
	BLO	neg	neg
	*pH	6.5	6.0
	PRO	neg	neg
	URO	0.2	0.2
	NIT	neg	neg
	LEU	neg	neg
Donor #3 male	COL	yellow	yellow
	CLA	clear	clear
	GLU	neg	neg
	BIL	neg	neg
	KET	neg	neg
	*SG	1.015	1.020
	BLO	neg	neg
	*pH	6.0	6.5
	PRO	neg	neg
	URO	0.2	0.2
	NIT	neg	neg
	LEU	neg	neg

Table 2. Urine samples preserved with the preservative reagent were compatible with automated urine analyzers. The performance of unpreserved original urine and preserved urine (urine 20:1 reagent) was compared on both urine chemistry analyzer CLINITEK Atlas® and urine sediment analyzer Sysmex UF-1000i™. The preservative reagent showed a minimal effect on automated urine analysis. *Only slight changes in pH, specific gravity, and conductivity were observed.

CONCLUSION

The novel urine preservative reagent can stabilize cfDNA and blood cells in urine and minimize the post-sampling urinary DNA background for an extended period of time at various storage temperatures ranging from 6 °C to 37 °C (previous temperature data not shown). Preserved urine samples are generally compatible with urine analyzers. This new methodology provides clinical laboratories with great flexibility and convenience in urine sample processing, handling and storage for urinary nucleic acid testing as it eliminates the necessity for immediate separation of supernatant after urine collection and refrigerating/freezing urine for transport. Reliable routine urinalysis results can be obtained even if there is a delay between collection and examination.

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