# Overcoming Challenges of cfDNA Analysis with Optimized Preanalytical Workflows Enables Complementary Urine and Blood Liquid Biopsy Studies

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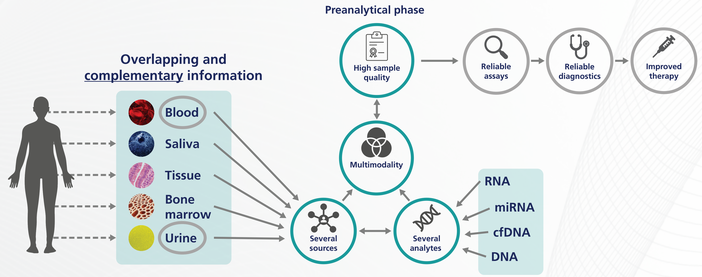
## Introduction

Urine and blood have the potential to provide complementary biomarker information and may be analyzed in parallel to increase sensitivity for disease detection in liquid biopsy research. Further studies are needed to support the value of complementary urine and blood analysis including the analysis of transrenal cell-free DNA (cfDNA).

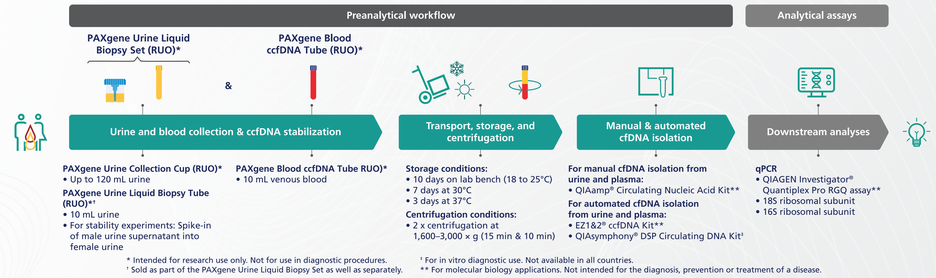
However, two main challenges previously impeded reliable urine cfDNA analysis. First, cfDNA degradation by nucleases leads to a loss of cfDNA targets of interest. Second, bacterial growth in urine contributes nucleases and DNA that dilutes the cfDNA of interest.

This study analyzed cfDNA yields, cfDNA kinetics and bacterial growth in unstabilized urine to investigate the challenges for urine cfDNA analysis and tested several urine and blood preanalytical workflows emphasizing blood stabilization and urine stabilization to optimize cfDNA analysis.




## Material and Methods

Urine and venous blood were collected from apparently healthy, consented individuals. Female urine was spiked with cell-free male urine supernatant. Blood was stabilized; urine was stabilized or left unstabilized. Urine and blood samples were stored at different conditions and lengths of time: e.g., for 3 days at 37°C, for 7 days at 30°C or for 10 days on the lab bench (18°C to 25°C). cfDNA was isolated from the urine supernatant or plasma using different automated and manual isolation kits. cfDNA was quantified by quantitative PCR (qPCR).




* Intended for research use only. Not for use in diagnostic procedures.
† Sold as part of the PAXgene Urine Liquid Biopsy Set as well as separately.
‡ For in vitro diagnostic use. Not available in all countries.
** For molecular biology applications. Not intended for the diagnosis, prevention or treatment of a disease.

## Results

### Stabilization of cfDNA in Urine

The study showed a loss of cfDNA in unstabilized urine by more than 90%. Integration of an innovative stabilization technology for urine cfDNA into the preanalytical workflow was found to minimize degradation and hence, to increase the cfDNA yield and stabilize urine cfDNA profiles for reliable analysis.

### Bacterial Growth in Urine

Bacterial growth was detected in unstabilized samples with an increase of more than 100× in 16S DNA content. Implementation of a novel stabilization technology in the preanalytical workflow effectively suppressed bacterial overgrowth and thereby prevented release of additional nucleases and bacterial DNA.

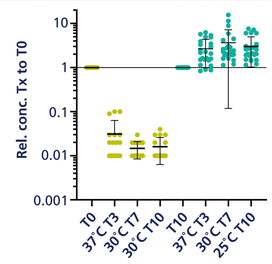



Figure 1: Total cfDNA quantification in unstabilized and PAXgene stabilized urine [mean ±SD, n=24]

Urine samples were collected in the PAXgene Urine collection cup and left unstabilized or were stabilized in the PAXgene Urine Liquid Biopsy Tube for indicated storage conditions. QIAsymphony was used for cfDNA isolation and qPCR for quantification of total yield relative to collection time point (<2 h).

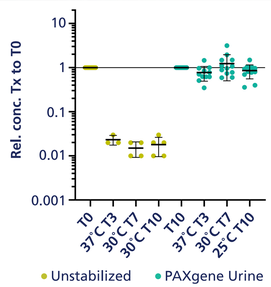



Figure 2: Quantification of cfDNA from spiked urine with and without PAXgene stabilization [mean ±SD, n=12]

Urine samples were collected in the PAXgene Urine collection cup and left unstabilized or were stabilized in the PAXgene Urine Liquid Biopsy Tube for indicated storage conditions. QIAsymphony was used for cfDNA isolation and qPCR for quantification of male spike-in relative to collection time point (<2 h).

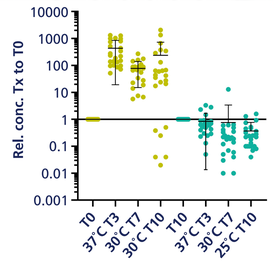



Figure 3: Bacterial growth quantified by 16S DNA content in unstabilized and PAXgene stabilized urine samples [mean ±SD, n=24]

Urine samples were collected in the PAXgene Urine collection cup and left unstabilized or were stabilized in the PAXgene Urine Liquid Biopsy Tube for indicated storage conditions. QIAsymphony was used for cfDNA isolation and qPCR for quantification of bacterial DNA relative to collection time point (<2 h).

### Extraction Comparability for Urine and Blood Samples

As isolation methods in conjunction with biospecimen collection and stabilization can affect cfDNA analysis, this study investigated manual and automated cfDNA isolation methods. All tested isolation procedures showed compatibility with the used stabilization technologies and allowed isolation of high-quality cfDNA.

### Extraction Linearity of Urine and Blood Samples

This study further investigated cfDNA isolation linearity for urine and blood for input volumes between 2 to 10 mL. All tested volumes showed compatibility with the used stabilization technologies for urine and blood regardless of the sample input volume and allowed isolation of high-quality cfDNA as analyzed by qPCR.

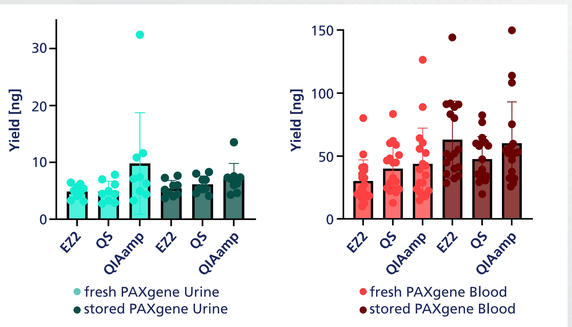



Figure 4: Comparison of different technologies for cfDNA extraction from PAXgene stabilized urine and blood samples

Extraction of cfDNA with manual, QIAamp Circulating Nucleic Acid Kit (QIAamp), and automated, EZ2 Connect and QIAsymphony (QS), methods from fresh or stored PAXgene stabilized urine and plasma samples with qPCR quantification (Investigator®, mean ±SD). Urine: spike-in, <4 h or 4 d, 10 mL input, n=9. Plasma: total cfDNA, <4 h or 10 d, 4 mL input, n=18.

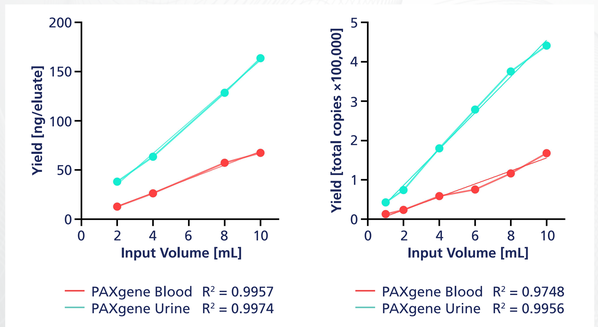


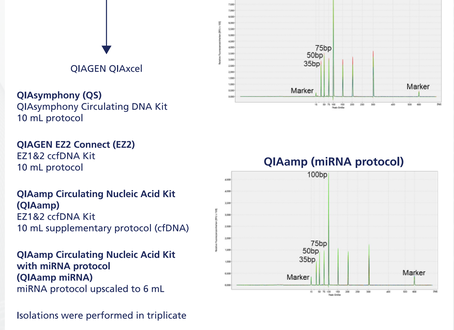

Figure 5: Linearity of different input volumes for EZ2 Connect and QIAsymphony based cfDNA extraction from PAXgene stabilized urine and blood samples [mean of n=12]

Extraction of cfDNA with EZ2 Connect (left; quantified with Investigator® qPCR) and QIAsymphony (right; quantified with 18S qPCR) from different PAXgene stabilized urine supernatant and PAXgene stabilized plasma input volumes.

### Isolation of transrenal DNA

Cell-free DNA that passes from the blood stream into urine through the kidneys can be referred to as transrenal DNA and differs in nature from cfDNA in blood. Transrenal DNA is not bound to nucleosomes and has a shorter fragment size.

To enable detection of transrenal DNA, the capabilities of different kits and instruments to extract small DNA fragments were analyzed. QIAsymphony and EZ2 Connect instruments and the manual QIAamp Circulating Nucleic Acid Kit extraction with miRNA protocol were able to purify DNA fragments down to 35 bp.




**QIAsymphony (QS)**
QIAsymphony Circulating DNA Kit
10 mL protocol

**QIAGEN EZ2 Connect (EZ2)**
EZ1&2 ccfDNA Kit
10 mL protocol

**QIAamp Circulating Nucleic Acid Kit (QIAamp)**
EZ1&2 ccfDNA Kit
10 mL supplementary protocol (cfDNA)

**QIAamp Circulating Nucleic Acid Kit with miRNA protocol (QIAamp miRNA)**
miRNA protocol upscaled to 6 mL

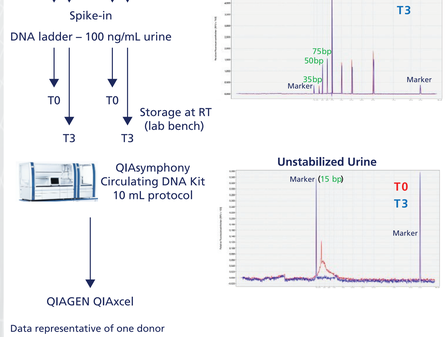
Isolations were performed in triplicate

Figure 6: Extraction of cfDNA fragments in length of transrenal DNA with different methods

A PBS-diluted DNA ladder was extracted with automated (QIAsymphony (QS) and EZ2) and manual (QIAamp Circulating Nucleic Acid Kit (QIAamp) miRNA protocol) cfDNA extraction methods and measured by capillary electrophoresis on a QIAxcel instrument.

### Detection and Stabilization of Transrenal DNA in Urine

After extraction methods for transrenal DNA were established (see Figure 6), the recovery of transrenal DNA from urine was analyzed. DNA fragments down to a size of 35 bp could be extracted from PAXgene stabilized urine after spike-in and after 3 days of storage at room temperature. The short fragments could not be recovered from unstabilized urine even shortly after spike-in (<2 h).




Data representative of one donor

Figure 7: Extraction of cfDNA fragments in length of transrenal DNA from urine

A DNA ladder with 35, 50, 75 bp and longer fragments was spiked into urine and extracted with a QIAsymphony instrument within 2 h or after 3 days of urine storage. Isolated cfDNA was measured by capillary electrophoresis on a QIAxcel instrument.

## Conclusion

In summary, this study emphasizes the critical importance of optimizing preanalytical workflows, including biospecimen stabilization, to ensure the reliability and reproducibility of cfDNA analysis. Dedicated and validated urine and blood cfDNA workflows were investigated that could enable exploring the complementarity of liquid biopsy biomarkers with the potential to improve research outcomes.

## Disclaimer



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