Application Note

Optimized urine liquid biopsy workflow: From sample collection to cfDNA stabilization and purification, ready for digital PCR analysis

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Introduction

Cell-free DNA (cfDNA) in urine is an avenue for liquid biopsy research. In addition to being a standalone source for biomarkers, urine cfDNA has been shown to provide complementary information to blood cfDNA. The advantage of urine is that it offers a truly non invasive collection method. Furthermore, urine cfDNA originates from cells of non-urological as well as genitourinary organs, having potential in a wide range of research fields, such as cancer or transplant research using state-of-the-art downstream technologies such as digital PCR (1).

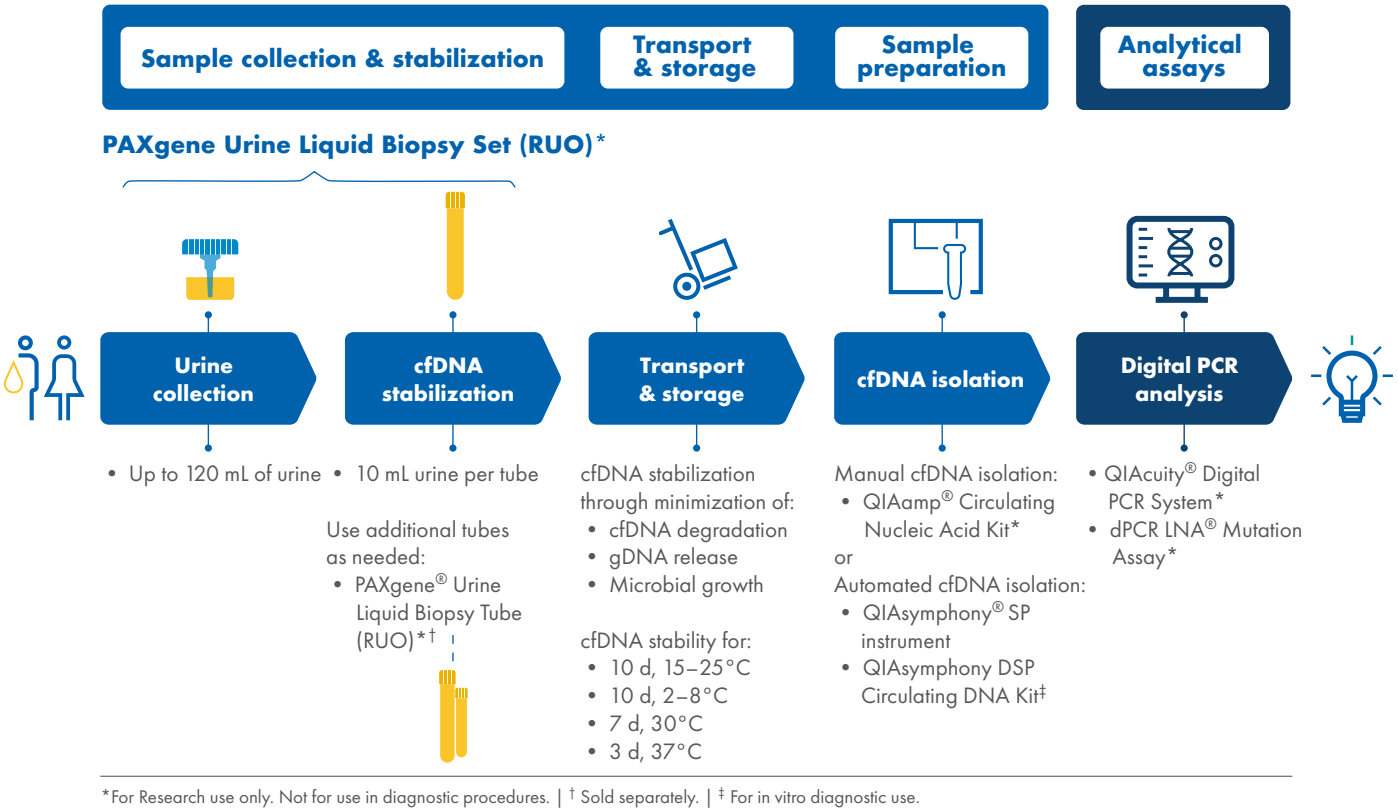

Figure 1: Urine liquid biopsy workflow.
The PAXgene Urine Liquid Biopsy Set* as part of a complete preanalytical workflow for biomarker detection by dPCR on the QIAcuity instrument.

Digital PCR (dPCR), as on the QIAcuity Digital PCR system, is a powerful technique that allows precise quantification of DNA, detection of mutations, analysis of copy number variation (CNV) and more.

However, reliable analysis with state-of-the-art downstream technologies is only possible with high-quality cfDNA samples as input material, which are the result of optimized preanalytical workflows.

Here, we describe a manual and an automated preanalytical workflow (Figure 1), starting from urine collection and cfDNA stabilization with the PAXgene Urine Liquid Biopsy Set to the isolation of high-quality unmodified cfDNA, allowing reliable urine cfDNA analysis with the QIAcuity Digital PCR system.

Materials and methods

Samples

Second urine of the day was collected in the PAXgene Urine Collection Cup, which is part of the PAXgene Urine Liquid Biopsy Set, by self-declared healthy volunteers at QIAGEN Hilden according to the *PAXgene Urine Liquid Biopsy Set handbook*. All donating individuals gave their written informed consent for sample collection.

Experimental setup

Where indicated, urine samples were spiked with a cfDNA standard containing PIK3CA mutations (Multiplex 5% allele frequency cfDNA standard, SensID), or urine from at least three individuals was mixed to generate a urine pool.

Urine cfDNA stabilization

Urine was stabilized by transfer from the PAXgene Urine Collection Cup into the PAXgene Urine Liquid Biopsy Tube or was left unstabilized. Where indicated, the stabilization solution was mixed with the urine by pipetting instead of usage of the set (PAXgene Urine Liquid Biopsy technology).

cfDNA isolation

Urine was centrifuged at room temperature (15–25°C) for 15 minutes at 1900 x *g*. The supernatant was centrifuged at room temperature (15–25°C) for 10 minutes at 1900 × *g*. cfDNA was isolated from the urine supernatant after the second centrifugation either automated on the QIAsymphony SP instrument using the QIAsymphony DSP Circulating DNA Kit or manually using the QIAamp Circulating Nucleic Acid Kit. Input volume for cfDNA isolation was 10 mL for urine from healthy individuals without spike-in (supplementary protocol for QIAamp cfDNA isolation from 10 mL urine stabilized in the PAXgene Urine Liquid Biopsy Tube available on the PAXgene Urine Liquid Biopsy Set product webpage) and 6 mL for urine from healthy individuals with spike-in. cfDNA isolation was performed either on the day of urine collection without any storage (T_0) or, where indicated, after 3 days of storage at the lab bench (room temperature, T_3).

Digital PCR analysis

dPCR was performed on the QIAcuity instrument using QIAcuity Multitarget LNA Mutation Detection Assay (PIK3CA H1047R GeneGlobe® cat. no. DMH0000036-A200) assessing the copy number of

wildtype *PIK3CA* as well as of the *PIK3CA* mutant H1047R. For samples from healthy individuals without spike-in 10 µL cfDNA eluate were used and 22 µL cfDNA eluate were used as input volume for the dPCR for samples from healthy individuals with spike-in. Preparation included an XbaI DNA digestion. dPCR was performed in a QIAcuity Nanoplate 26k 24-well. gDNA mixed with gBlocks harboring the PIK3CA H1047R mutation (IDT, Newark, USA) was used as a positive control, and dH_2O was used as a negative control. Data analysis was performed using the QIAcuity Software Suite version 2.5.0.0 and 2.5.0.24 (healthy individuals without spike-in). Where indicated, cfDNA was quantified in triplicates.

Results and discussion

Urine cfDNA isolated from the PAXgene Urine Liquid Biopsy Set was compatible with dPCR (QIAcuity)

cfDNA can include information on the entire genome. To test the dPCR compatibility of urine cfDNA isolated from the PAXgene Urine Liquid Biopsy Tube, the detection of one example gene, *PIK3CA*, using the QIAcuity was performed.

For all tested urine cfDNA eluates, QIAamp and QIAsymphony eluates from eight different urine pools, detection of PIK3CA was possible (Figure 2). These data indicate compatibility of the urine cfDNA eluates with dPCR. The 1D scatter plot (Figure 3) confirms the compatibility of the urine cfDNA eluates with dPCR on the QIAcuity. No signs of dPCR inhibition through the urine cfDNA eluates could be observed. Inhibitory effects would be visible as V-shapes in the 1D scatter plot (2).

Additionally, data shows that both manual and automated cfDNA purification methods yielded consistent and comparable cfDNA yields.

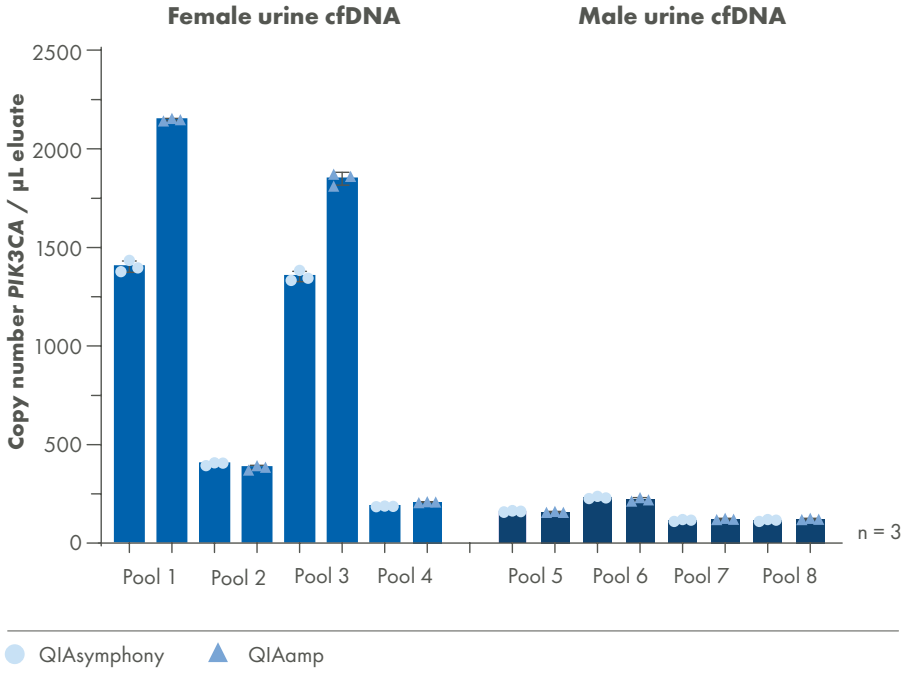

Figure 2: dPCR analysis.
Copy numbers of wildtype PIK3CA/µL of cfDNA eluates isolated either with the QIAsymphony DSP Circulating DNA Kit or the QIAamp Circulating Nucleic Acid Kit from urine stabilized with the PAXgene Urine Liquid Biopsy Tube. dPCR was performed using the LNA PIK3CA Assay DMH0000036 on the QIAcuity instrument. Data include 8 urine pools with 3 qPCR replicates, n = 24. Mean and SD are denoted.

Figure 2 further shows that the yield of cfDNA isolated from 10 mL urine stabilized with the PAXgene Urine Liquid Biopsy Set is sufficient for dPCR analysis. Expected differences in copy numbers were detected. The concentration of cfDNA in urine varies considerably, influenced by many factors, including among different individuals, as shown in Figure 2, between different urine pools, or sex, with a lower concentration of urine cfDNA in urine from male individuals. Further influencing factors can be the health status of the donating individual, the time of collection and the hydration status.

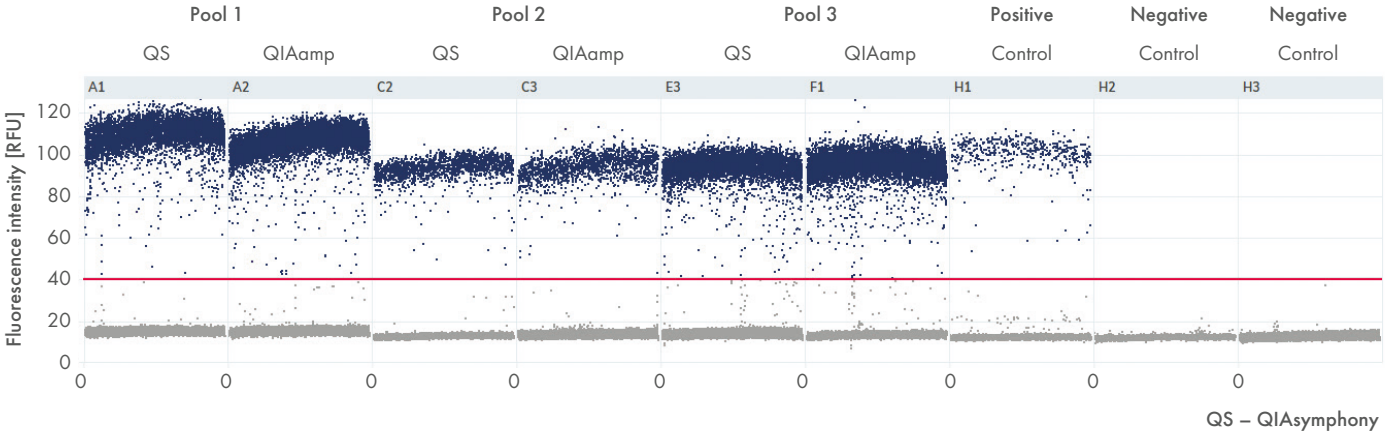

Figure 3: dPCR compatibility.
A 1D scatter plot generated by the QIAcuity Software Suite, exemplarily showing data for pools 1, 2 and 3, a positive control and two negative controls (water).

The PAXgene Urine Liquid Biopsy Set stabilized urine cfDNA, allowed urine storage and enabled reliable mutation detection by dPCR

cfDNA can originate from tumor cells making it a valuable analyte in liquid biopsy research. This cell-free tumor-derived DNA (ctDNA) harbors aberrations, such as mutations, reflecting the aberrations in the genome of the tumor cells. An example for a well-known cancer-related mutation is the *PIK3CA* H107R mutation known in breast cancer.

To test whether cfDNA isolated from the PAXgene Urine Liquid Biopsy Set is compatible with dPCR mutation detection, a cfDNA spike-in including the *PIK3CA* H107R mutation was introduced into unstabilized urine from healthy individuals (Note: Varying nuclease activity in urine from different individuals inducing varying degrees of degradation of cfDNA, including the spike-in, that can lead to differences in the total yield of detected spike-in in this experiment). Urine cfDNA was either left unstabilized or was stabilized with the PAXgene Urine Liquid Biopsy technology. cfDNA was isolated either on the day of urine collection (T_0) or after storage for 3 days at room temperature (15–25°C on the lab bench; T_3).

Stabilization with the PAXgene Urine Liquid Biopsy technology increased the mean copy number of *PIK3CA* WT as well as H1047R that could be detected in urine by dPCR on the QIAcuity when compared to unstabilized urine samples (Figure 4). This effect was already found for cfDNA isolated on the day of urine collection and was even more prominent after urine storage for three days. After three days of unstabilized urine storage, almost no cfDNA and no mutation could be detected, whereas stabilized samples allowed reliable mutation detection, even after storage (Figure 4).

Data indicate the need for urine stabilization to provide sufficient input cfDNA for dPCR and to stabilize the cfDNA profile over the time of urine storage to allow reliable cfDNA analysis.

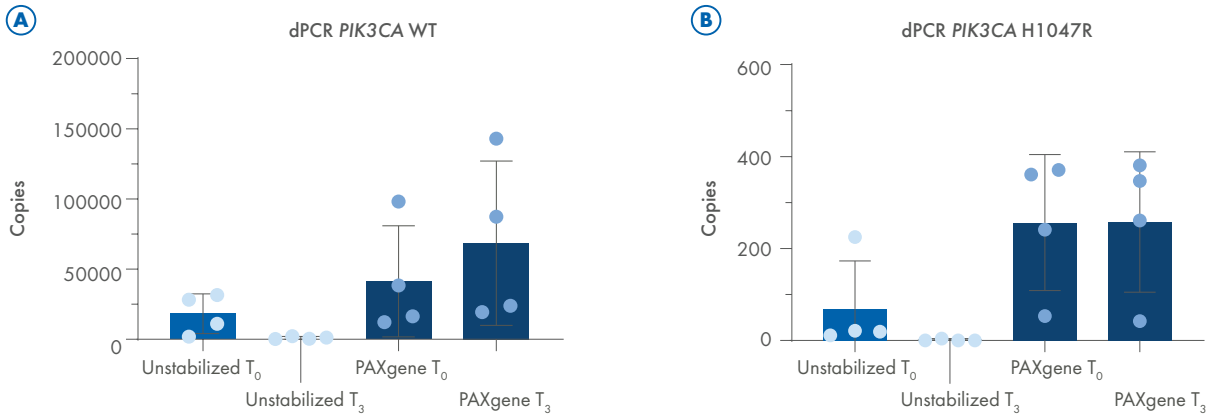

Figure 4: Mutation detection compatibility.
Copy number of WT PIK3CA [A] and PIK3CA H1047R mutation [B] in cfDNA eluates isolated with the QIAsymphony DSP Circulating DNA Kit from unstabilized urine or urine stabilized with the PAXgene Urine Liquid Biopsy technology before (urine processed within 4 hours of urine collection (T_0, set to 1)) and after urine storage (T_3, 3 days at 15–25°C). Urine from healthy individuals was spiked with a mutation harboring cfDNA standard. Data include urine from 4 urine pools, n =4. Mean and SD are denoted.

Conclusion

This study showcases an optimized, end-to-end urine liquid biopsy workflow – from urine collection to cfDNA stabilization and isolation, ready for dPCR analysis. The workflow presented helps maximize the analytical potential of urine, helping reveal biomarkers such as mutations of interest, while bypassing common bottlenecks. The study underscores the importance of cfDNA stabilization for urine storage and accurate downstream analysis. The PAXgene Urine Liquid Biopsy Set, integrated into an automated or manual optimized preanalytical workflow for urine cfDNA analysis, facilitates reliable urine storage and dPCR-based biomarker detection by overcoming the challenge of loss and dilution of the cfDNA target of interest in urine. The importance of isolation methods – either automated via the QIAsymphony SP instrument or manually via QIAamp technology – that contribute to high-quality DNA, free of contaminants or inhibitors, is also highlighted. The study also demonstrates the reliability of the QIAcuity Digital PCR System when it comes to detection and absolute quantification of mutant cfDNA copies in a high WT background.

Ordering information

Product	Cat. no.
PAXgene Urine Liquid Biopsy Set*	769143
PAXgene Urine Liquid Biopsy Tube*	769114
QIAamp Circulating Nucleic Acid Kit*	55114
QIAsymphony SP instrument†	9001297
QIAsymphony DSP Circulating DNA Kit‡	937556
QIAcuity Digital PCR System*§	Various
dPCR LNA Mutation Detection Assay*	250200
	Configure at GeneGlobe
QIAcuity Probe Master Mix QIAcuity Probe PCR Kit*	250101

* For Research Use Only. Not for use in diagnostic procedures.

† The QIAsymphony SP instrument is intended for use by professional users, such as technicians and physicians trained in molecular biological techniques and the operation of QIAsymphony SP instrument. The QIAsymphony SP instrument is designed to perform automated purification of nucleic acids in molecular diagnostic and/or molecular biology applications. It is intended to be used only in combination with QIAsymphony Kits indicated for use with the QIAsymphony SP for the applications described in the kit handbooks.

‡ The QIAsymphony DSP Circulating DNA Kits are intended for in vitro diagnostic use.

§ QIAcuity is intended for molecular biology applications. This product is not intended for the diagnosis, prevention or treatment of disease.

References

1. Su YH, et al. Human urine contains small, 150 to 250 nucleotide-sized, soluble DNA derived from the circulation and may be useful in the detection of colorectal cancer. J Mol Diagn. 2004;6(2):101-7.
2. https://www.qiagen.com/products/discovery-and-translational-research/pcr-qpcr-dpcr/dpcr-assays-kits-and-instruments/dpcr-kits/q-solution-kit

Scan the QR code or use the link to learn more: **www.qiagen.com/cfDNA**

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN or PreAnalytiX kit instructions for use or user manual. QIAGEN or PreAnalytiX kit instructions for use and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.