Cell-free DNA preservation and extraction from urine using Novosanis Colli-Pee® UAS™ collection device and nRichDX Revolution System™

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Urine samples collected at home using the Novosanis Colli-Pee® device. Urine samples, with and without UAS™ preservative, were extracted using Revolution System™ (nRichDX) or the QIAamp™ Circulating Nucleic Acid Kit (QIAGEN). Compared to samples extracted with QIAamp, Revolution System gave higher cell-free DNA yield (due to both higher extraction efficiency per mL of urine and larger input volume) and thus, higher biomarker detection sensitivity. UAS preservative demonstrated the ability to preserve both endogenous urinary cell-free DNA and spiked mono-nucleosomal DNA and was found to be compatible with both extraction systems. Based on the study performed, Colli-Pee® with UAS preservative, combined with the semi-automated, cfDNA extraction platform Revolution System, offers a highly efficient workflow for large-scale urine sample collection, preservation and downstream processing with higher cell-free DNA yield than the alternative extraction system evaluated.

Keywords: first-void urine, cell-free DNA (cfDNA), liquid biopsy, urine collection and preservation, Colli-Pee®

Introduction

Cell-free DNA (cfDNA) is commonly utilized as an analyte during liquid biopsy analysis in various clinical fields, especially oncology and prenatal diagnosis.[1] Urine collection, being non-invasive, has an obvious advantage over blood for liquid biopsy purposes.[2] However, the urine environment (capacity for microbial growth and presence of nucleases) and the labile nature of intact host cells at the point of sample collection drives the need for proper preservation and processing of urine samples. Moreover, isolating cfDNA from biological fluids is challenging, due to its low concentration in the sample, especially in urine. These factors are of particular concern, as studies around urinary analytes for cancer diagnosis, monitoring of cancer progression and therapeutic effects are rapidly expanding in cohort sizes.[3] Multisite, at-clinic collections are increasingly prohibitive for large-scale recruitment and lead to variability in the time between sample collection and downstream processing. A means of standardized volumetric sample collection and preservation coupled with an efficient extraction system is needed to address and mitigate these known issues. Novosanis offers standardized volumetric first-void urine collection with Colli-Pee® UAS™ collection devices (Colli-Pee collection devices prefilled with the UAS preservative) for analyte preservation to facilitate home-based sampling. UAS preservative was developed to be agnostic for different extraction methods. Here, we have investigated and compared the extraction efficiency and compatibility of semi-automated cfDNA extraction platforms — Revolution System™ (nRichDX) and the QIAamp Circulating Nucleic Acid Kit (QIAGEN) — for the isolation of cfDNA from the random first-void urine samples collected using Colli-Pee (Novosanis) and in the absence and presence of UAS preservative.

Materials and methods

Sample collection and processing: First-void urine samples were collected from healthy female and male donors with the Colli-Pee device (Colli-Pee® FV-5020/Colli-Pee® FV-5040; Novosanis). In one of the studies, pooled urine samples (n = 6; 3 male

pooled and 3 female pooled) had 1 aliquot mixed with UAS preservative; the other was left unpreserved with no preservative addition (NA). Both types of urine sample were stored for 7 days at room temperature (RT: 23°C ± 3°C). On day 0 (T0) and day 7 (T7), 25 mL aliquots of each unpreserved and preserved sample were centrifuged at 3000×*g* for 10 minutes at RT. Precleared supernatants from T0 and T7 time points were stored at -80°C until extraction. In another study, 3 pooled urine samples (2 male pooled and 1 female pooled) had 1 aliquot mixed with UAS preservative; the other was left unpreserved with no preservative addition (NA). Both types of urine sample were spiked with 10 ng/mL of mononucleosomal DNA (mnDNA) extracted from the human lung cancer cell line (NCI H441). The spiked mnDNA contains the known KRAS G12V mutation. Both types of urine sample were stored for 7 days at RT. On day 0 (T0) and day 7 (T7), 5 mL aliquots of each unpreserved and preserved sample were stored directly at -80°C until extraction.

Cell-free nucleic acids extraction: 4 mL and 20 mL aliquots of precleared supernatant from each unpreserved sample and sample containing UAS preservative underwent cell-free nucleic acids (cfNAs) extraction using the QIAamp Circulating Nucleic Acid Kit (QIAGEN) and Revolution System (nRichDX), respectively, according to the manufacturer's instructions. In the second study, 5 mL frozen aliquots from both T0 and T7 time points were thawed, centrifuged and the obtained supernatants were batch extracted for cfNAs using Revolution System. The extracted cfNAs profile was assessed on 4200 TapeStation System (Agilent) using cfDNA ScreenTape, according to the manufacturer's instructions.

Human β-globin qPCR assay: Extracted nucleic acids from urine samples were subjected to a qPCR assay for the quantification of human cfDNA content using 2X iTaq Universal SYBR Mastermix (Bio-Rad). The primers and the PCR conditions of the human β-globin qPCR assay are described in the literature.[4] Human genomic DNA (gDNA) standards with serial dilution (1, 1:10, 1:100, 1:1000) and a non-template control (2 µL of RNase/DNase-free water) were used in each qPCR run. Human cfDNA quantity was assessed using C_t values obtained from the qPCR assay. ΔC_t values were calculated and used as a measure of preservation. "ΔC_t" stands for [C_t(T7)-C_t(T0)]. "C_t(T0) and C_t(T7)" stands for the qPCR cycle threshold at day 0 and day 7. Two-tailed p-values ($p \leq 0.05$) from the Wilcoxon signed-rank test were used as the statistical measure. Actual median ΔC_t values obtained were compared to a hypothetical median value of $\Delta C_t = 0$ to determine the efficiency of UAS preservative for urinary analytes preservation. Median $\Delta C_t = 0$ reflects absolute analyte preservation.

KRAS G12V mutation qPCR assay: Extracted nucleic acids containing the KRAS G12V mutation from urine samples were subjected to a qPCR assay to quantify the KRAS G12V mutation using TaqMan Genotyping MasterMix (Thermo Fisher). The primers and the PCR conditions for the KRAS G12V qPCR assay are described in the literature.[5] A standard curve was created using a KRAS G12V reference standard, 50% 1 µg (Horizon Discovery). A positive control containing the KRAS G12V mutation and a non-template control (3 µL of nuclease-free water) were used in each qPCR run. A QuantStudio™ 3 Real-Time PCR System (Thermo Fisher) was used to perform the PCR reactions. Quantification of KRAS G12V mutation was assessed using C_t values obtained from the qPCR assay

Results

Figure 1A illustrates an increase in ΔC_t values (median ΔC_t: +6.6 to +7.8; p-value: 0.03) for β-globin DNA, demonstrating a statistically significant decrease in human cfDNA content after the unpreserved samples were stored for 7 days at RT. In contrast, there was no change in ΔC_t values (median ΔC_t: -0.3 to -0.4; p-value: 0.16) for β-globin DNA levels, suggesting no change in human cfDNA content in samples containing UAS preservative held for 7 days at RT (Figure 1A). Both QIAamp and Revolution System extracted cfDNA showed similar observations for the preservation changes in the unpreserved (NA) and samples containing UAS preservative (Figure 1A). However, Revolution System showed lower C_t values (median C_t: 27-28), indicating the presence of higher

cfDNA content relative to QIAamp extraction method, which showed median C_t values ranging from 30 to 33 for the T0 baseline and samples containing UAS preservative (Figure 1B). Lower C_t values correspond to higher concentration and vice versa.

Figure 2A illustrates an increase in C_t values (median C_t: 45; maximum cycle threshold as no amplification was obtained after 45 cycles in qPCR reactions) for spiked mnDNA with KRAS G12V mutation, demonstrating an observable loss in the unpreserved samples stored for 7 days at RT. Even at T0 time point, unpreserved samples showed higher C_t value (median C_t: 32.9) and loss of spiked mnDNA relative to sample containing UAS preservative (median C_t: 28.8) (Figure 2A). In contrast, there was no statistically significant change in C_t values between T0 (median C_t: 28.8) and T7 (median C_t: 29.1) time points for spiked mnDNA, suggesting effective preservation in samples containing UAS preservative held for 7 days at RT (Figure 2A). In fact, samples containing UAS preservative showed full recovery (obtained C_t value is equivalent to mnDNA reference C_t value of 29) of spiked mnDNA under freeze-thaw and long-term storage at RT conditions. Unpreserved (NA) urine samples showed loss of spiked mnDNA profile under freeze-thaw and long-term storage at RT conditions, while samples containing UAS preservative showed effective preservation of cfDNA profiles under these conditions (Figure 2B).

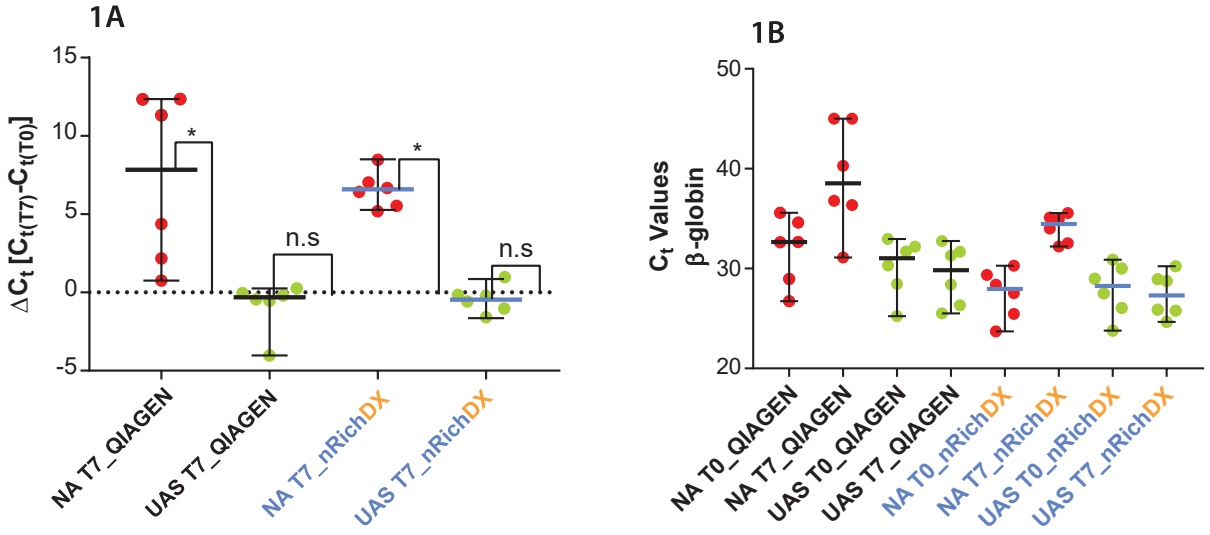

***Figure 1A**. β-globin qPCR assay showed loss of human cfDNA content (median ΔC_t: +6.6 to +7.8; p-value: 0.03) for T7 time point in unpreserved samples unlike samples containing UAS preservative, which showed effective cfDNA preservation with no significant change (median ΔC_t: -0.3 to -0.4; p-value: 0.16). T0 and T7 stand for day 0 and day 7 time points. Dotted lines (…) correspond to $\Delta C_t = 0$. N.s. stands for non-significant.*

***Figure 1B.** β-globin qPCR assay showed lower C_t values for the cfDNA extracted using Revolution System (nRichDX) as compared to the QIAamp Circulating Nucleic Acid Kit (QIAGEN). The horizontal bar represents the median with 95% confidence interval (CI).*

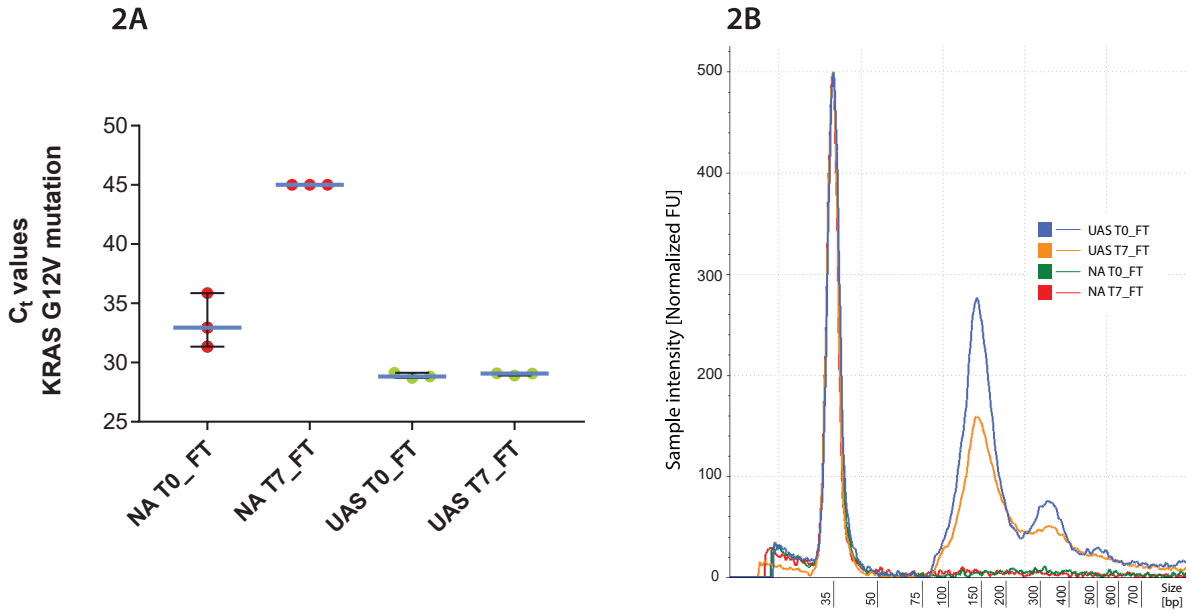

***Figure 2A**. KRAS G12V mutation qPCR assay showed no amplification (median C_t:45; maximum cycle threshold as no amplification was obtained after 45 cycles in qPCR reactions) and drastic loss of spiked mnDNA in unpreserved samples under freeze-thaw (FT) and 7 days storage at RT conditions, unlike samples containing UAS preservative, which showed effective cfDNA preservation with no statistically significant change in C_t values. T0 and T7 stands for day 0 and day 7 time points. The horizontal bar represents the median with 95% confidence interval (CI).*

***Figure 2B.** Representative TapeStation data for spiked mnDNA. Complete loss of spiked mnDNA signal was observed in unpreserved samples, unlike samples containing UAS preservative, which showed effective preservation and detection of spiked mnDNA profile, under FT and 7 days storage at RT conditions.*

Conclusions

Revolution System (nRichDX) for cfDNA isolation and the QIAamp Circulating Nucleic Acid Kit (QIAGEN) successfully extracted cfDNA from urine samples, with and without UAS preservative, collected using the Colli-Pee device. cfDNA isolated from large urine volumes (20 mL) in a single extraction using Revolution System showed higher yield and detection sensitivity. Larger input volumes along with higher extraction efficiency of Revolution System provides higher cell-free nucleic acids yield as shown previously for plasma and urine samples.[6,7] Due to the maximum sample input volume constraint, the QIAamp Circulating Nucleic Acid Kit was able to extract from smaller urine volumes (4 mL) with relatively lower yields and detection sensitivity compared to extractions from the nRichDX method. As the UAS preservative was developed to be agnostic in relation to different extraction methods, it is compatible with Revolution System, as shown by the preservation of endogenous β-globin cfDNA and spiked mnDNA quantity and quality (Figure 1A, 2A and 2B). In addition, Colli-Pee urine collection devices, combined with Revolution System, allow the flexibility to perform whole-sample extractions for cell-free nucleic acids. Overall, the first-void urine collection Colli-Pee device and UAS preservative from Novosanis, combined with Revolution System (nRichDX), a semi-automated cfDNA extraction platform, offers a seamless and efficient workflow for large-scale studies requiring urine sample collection, preservation, handling and downstream processing.

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