Why urine preservation is needed for molecular cancer biomarker detection

Amit Arora[1], Stephanie Jordaens[2], Arya Mehta[2], David Faster[2], Michelle Laeremans[2], Sanne Bruyninckx[2], Danielle Pasmans[2], Evgueni Doukhanine[1], Koen Beyers[2], Vanessa Vankerckhoven[2]
1 | DNA Genotek, Ottawa 2 | Novosanis, Belgium
October 2020

INTRODUCTION

Traditionally, cancer detection and treatment monitoring primarily rely on tumor analysis through tissue biopsies, a highly invasive and time-intensive method. In recent years, the development of minimally invasive procedures such as liquid biopsies, which analyze biomarkers in bodily fluids such as, blood, urine and saliva has opened new frontiers in cancer diagnostics[1].

A liquid biopsy involves extraction and analysis of genetic material from tumor cells, such as cell-free DNA (cfDNA), RNA (miRNAs, lncRNAs and mRNAs), proteins, peptides, and exosomes that shed into bodily fluids[2]. These biomarkers represent genetic and epigenetic events associated with cancer development and progression, potentially allowing for early detection, monitoring and prediction of therapy response in cancer patients[3].

The most common used liquid biopsy is blood; however, blood has limitations. It contains a high and complex protein repertoire, and blood sampling is relatively invasive, as well as poses risk to the patient and healthcare professional[4]. Alternatively, urine as a liquid biopsy offers several benefits. Urine sampling is quick and non-clinician dependent; moreover, urine can be collected in large quantities, providing sufficient material for multiple assays. However, there are challenges with urine collection. Given the possibility of microbial proliferation in urine as well as nuclease activity, the need to have methods to preserve collected urine samples is clear.

This white paper focuses on urine as a sample type and the importance of urine preservation.

URINE AS A SAMPLE TYPE

Urine Components

Human urine consists primarily of water, with organic and inorganic solutes. Consequently, urine represents a valuable source of biomarkers for the study of pathologies as cellular and cell-free material can be directly released into urine. Urine from pregnant women is also a useful source of fetal DNA that is present as cell-free DNA for non-invasive prenatal diagnostic and prognostic tests[5].

Urine Fractions

There is no such thing as a standard urine sample; the urine fraction, along with the timing of the specimen collection play an important role in sample quality. First-void urine, also known as first-catch or first-pass urine is collected at any time of the day and is typically referred to the first 20 mL of urine flush[6]. In particular, for sexually transmitted infections, first-void urine contains more DNA and RNA particles, as well as other analytes than other fractions such as a random or midstream urine sample[7].

Additionally, for urological cancers, urine is in many situations the preferred liquid biopsy source because it contains exfoliated tumor cells and cell-free tumor DNA and can be obtained easily, non-invasively, and repeatedly[8]. Compared to blood, urine is thought to be a more sensitive alternative for early detection or monitoring recurrence of cancers in the genitourinary tract[9]. Studies have also identified specific DNA, RNA, proteins, and metabolites for prostate cancer in first-void urine[10].

URINARY ANALYTES

Depending on the context and application in which a urine sample is taken, different analytes are investigated. Several urinary solutes have been established as a target for screening, diagnosis, prognosis and monitoring of disease as well as therapy effectiveness. Below is a list of analytes in urine that have been described in several publications in the field of oncology:

Cellular, cell-free and hypermethylated DNA

Most of the work so far focuses on cfDNA circulating in the bloodstream; however, for urological cancers, urine is a strong alternative to blood sampling, as it is easy, repeatable, and contains exfoliated (cancer) cells from different sites in the urinary system. Urine can contain DNA from these exfoliated cells, as well as cell-free DNA. Urinary cell-free DNA (UcfDNA) enters urine either from cells shedding from the urogenital tract, or cfDNA in circulation which passes through glomerular filtration. UcfDNA is believed to have a great potential as a non-invasive type of liquid biopsy[11].

As a large portion of DNA comes from non-cancerous human cells, DNA biomarkers that offer a high specificity for (pre-)malignancy are the most useful, reliable and can possibly be detected by highly sensitive PCR based detection technologies. Of these DNA alterations, hypermethylated DNA events are the most interesting targets as they are common in most cancers and are considered early events in tumor formation[8].

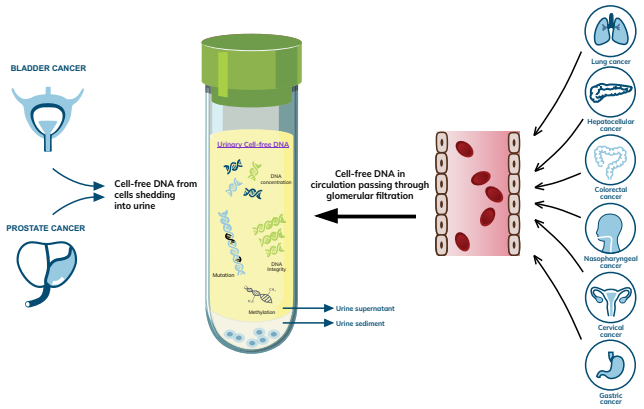

Figure 1: Urinary cell-free DNA (UcfDNA) adapted from Lu et al. 2017.[11]

Extracellular vesicles (EVs), RNA and proteins

Alongside cells and DNA, urine also contains vesicles that enter from the kidney and urinary tract. These urinary extracellular vesicles (UEVs) are lipid enclosed structures that can be roughly categorized in three categories, based on their formation mechanisms: (i) exosomes (ii) microvesicles (both originate from the plasma membrane, however, in a different fashion), and (iii) apoptotic bodies (originate from endosomes, and are shed into the urine by dying cells). UEVs contain various intracellular components (e.g. RNA and proteins) and are representative of the physiological condition of the cell from which they originated. As such, UEVs offer a 'snapshot' view of the life of a cell and therefore have a great potential as a platform for biomarker discovery. Additionally, secreted vesicles can play a role in inter-cellular communication as they are loaded with miRNA, mRNA and tRNA. Consequently, UEVs are an ideal candidate for research into disease progression and can even be used as potential therapeutic elements in the treatment of kidney and urinary tract disorders[12,13].

URINE PRESERVATION AND STABILITY

Despite a growing interest in urine as a sample type, especially for oncology and prenatal diagnosis, only a few studies on sample handling have been reported to date and no clear analytical consensus is available.

The required stability of a urine sample depends greatly on the type of analysis to be performed. The addition of preservative allows substantially longer preservation of urine and confidence in the utility of its content for diagnostic purposes.

For example, while hypermethylated DNA can be a suitable biomarker for cancer detection, the use of urinary hypermethylated DNA in clinical practice is constrained given the challenges of preserving urinary nucleic acids. Hence, urine needs to be stored and transported in a way that allows nucleic acid preservation for downstream analysis[14].

What is a Ct value?

An RT-PCR assay indicates the presence of a target sequence in a sample mixture through a fluorescent signal. The threshold cycle or Ct value is the RT-PCR cycle number during which fluorescent signal crosses the threshold of the background signal. However, a certain level of background fluorescence will always remain even in the absence of the target sequence.

- LOW Ct VALUES - *Lower Ct values can indicate high amounts of the target sequence within the sample*
- HIGH Ct VALUES - *Higher Ct values can indicate absence of the target sequence or suboptimal environmental conditions within the sample*

To understand the need for urine preservation, we compared the stability of several analytes in unpreserved urine.

Figure 2 shows the Ct values for a human β-globin and a bacterial 16S rRNA target gene in unpreserved urine samples left at room temperature for a period of 4 to 7 days.

Cell-free DNA was extracted from the urine samples using the QIAamp Circulating Nucleic Acid Kit (Qiagen), after which the human β-globin content was determined as per Jung et al. (2003)[15], and the bacterial 16S rRNA content was determined using an in-house extraction and amplification protocol (DNA Genotek, unpublished data).

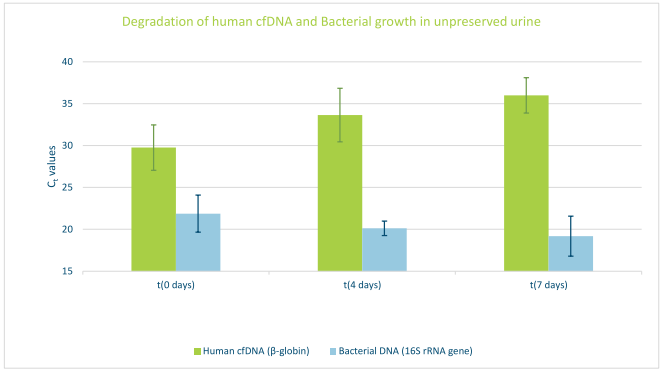

Figure 2: human β-globin and bacterial 16S rRNA detection in unpreserved urine. Significant differences observed between human β-globin and bacterial 16S rRNA (t-test p values of 1.25x10-6, 0.002 and 8.79x10-9, 0 days, 4 days and 7days, respectively).

The results show two different trends.

For human cfDNA, an increase in Ct values is observed after 4 and 7 days, suggesting lower concentrations of human cfDNA over time. This indicates that without preservation, human cfDNA in urine primarily degrades by nuclease activity. For bacterial 16S DNA, a decrease in Ct values is observed after 4 and 7 days, suggesting higher bacterial DNA content over time. This indicates bacterial (over)growth in urine. Additionally, the significantly lower Ct value for the bacterial DNA compared to the human cfDNA also implies that the latter could risk being diluted in the total mixture, potentially limiting detection if the downstream analysis method is chosen without due consideration.

The degradation of human cfDNA can also be observed in Figure 3, which shows tape station analysis results on day 0 and day 7. DNA fragments with an average fragment length of 198 bp and 445 bp are degrading to fragment lengths of 121 bp and to very small fragmented DNA (+/- 15 bp), indicating heavy nuclease activity in the unpreserved urine sample.

The extracted cell-free nucleic acids profile was assessed on the 4200 Agilent TapeStation platform (Agilent) according to manufacturer's instructions.

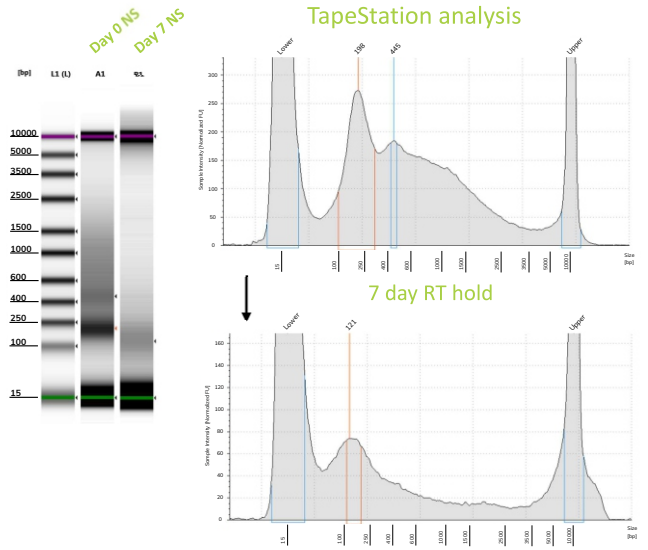

Figure 3: TapeStation analysis – human cfDNA

Similar trends can be seen when looking at the stability of several other analytes in urine when not in the presence of a preservative. For example, increasing Ct values were observed over a period of 7 days at room temperature, indicating a loss of stability and detectability, for cellular RNA, cell-free and EV RNA (Figure 4), as well as for Human Papillomavirus (HPV) plasmid DNA (Figure 5).

Urine EV RNA extraction was performed using exoRNeasy Maxi Kit (Qiagen), and the RNA contents were determined using the β-actin Taqman assay (ThermoFisher), after converting the RNA into cDNA using ThermoFisher Reagents, using an in-house protocol (DNA Genotek, unpublished data).

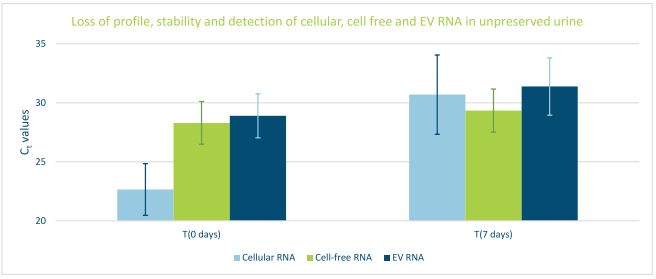

Figure 4: Loss of stability and detection of cellular, cell-free and EV RNA. Significant differences observed after 7 days for cellular RNA (t-test p value of 0.007), for cfRNA and EV RNA more data is required to investigate whether the trend will result in significant differences.

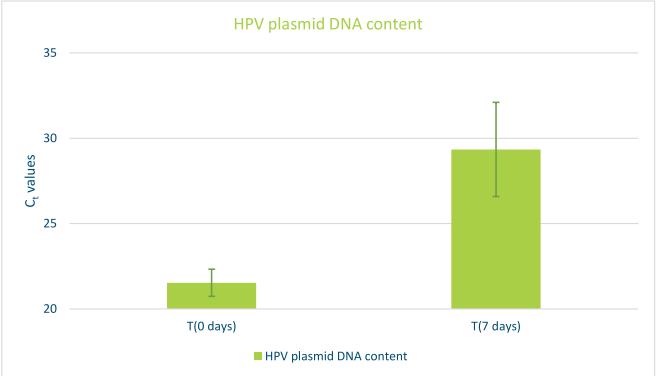

Figure 5: Degradation of HPV plasmid DNA. Significant differences observed after 7 days (t-test p value of 0.01)

To understand the effect of a preservative on urine samples, an experiment was set up using contrived urine samples that had been spiked with a known amount of HPV DNA.
In this setup, the following sample types were processed: (1) unpreserved urine samples, (2) urine samples preserved for 7 days in fresh Urine Conservation Medium (UCM) and (3) urine samples preserved in UCM lots that had been previously subjected to extreme temperature and relative humidity conditions, mimicking possible transportation scenarios (two different lots were used, named ISTA 3A UCM buffer lot 1 and 2, respectively).

All samples were analyzed on Cobas 4800/6800 systems, using the Cobas HPV test (Roche).

Figure 6 shows that for unpreserved samples, a significant increase in Ct values was observed after 7 days of storage. These results show that UCM can preserve HPV DNA in urine. Furthermore, no significant differences were observed in between the fresh preservative and the preservative lots that were exposed to the extreme environmental conditions. This indicates that transport does not impact the performance of the preservative.

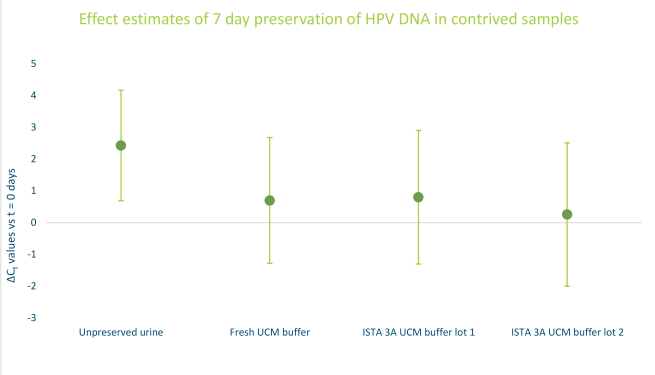

Figure 6: Urine preservation in UCM – Urine Conservation Medium.

CONCLUSION

Urine contains useful and diagnostically relevant biomarkers; however, analytes representing these biomarkers are susceptible to enzymatic and chemical degradation[16]. Additionally, in the period between collection and processing, rapid microbial (over)growth can be observed. Therefore, for samples that cannot be processed immediately after collection, the use of cold-chain transportation or a urine preservation medium is critical to maintain the quality of the collected samples.

Development of a urine preservative, which can maintain the original composition and integrity of a sample can open new avenues for biomarker detection in urinary liquid biopsy analysis. Furthermore, the use of a preservative has the potential to allow for improved storage and transport of urine at room temperature, allowing for home-based collection. Currently, Colli-Pee® can be prefilled with Urine Conservation Medium (UCM), to allow preservation of DNA in urine. To extend the preservation coverage to other analytes (RNA, EV, cfDNA) a new preservative, Urinary Analyte Stabilizer (UAS) is currently in development.

Contact Novosanis to know more about UAS and sample availability.

References:

(1) Wu, X., L. Zhu, and P.C. Ma, Next-Generation Novel Noninvasive Cancer Molecular Diagnostics Platforms Beyond Tissues. Am Soc Clin Oncol Educ Book, 2018. 38: p. 964-977.
(2) Di Meo, A., et al., Liquid biopsy: a step forward towards precision medicine in urologic malignancies. Mol Cancer, 2017. 16(1): p. 80.
(3) Cerchietti, L. and A. Melnick, DNA Methylation-Based Biomarkers. J Clin Oncol, 2017. 35(7): p. 793-795.
(4) Nolen, B.M. and A.E. Lokshin, -The advancement of biomarker-based diagnostic tools for ovarian, breast, and pancreatic cancer through the use of urine as an analytical biofluid. Int J Biol Markers, 2011. 26(3): p. 141-52.
(5) Tsui, N.B., et al., High resolution size analysis of fetal DNA in the urine of pregnant women by paired-end massively parallel sequencing. PLoS One, 2012. 7(10): p. e48319.
(6) Wisniewski, C.A. et al., Optimal Method of Collection of First-Void Urine for Diagnosis of Chlamydia trachomatis Infection in Men. J Clin Microbiol, 2008. 46 (4): p. 1466-1469.
(7) De Baetselier, I., et al., Evaluation of the 'Colli-Pee', a first-void urine collection device for self-sampling at home for the detection of sexually transmitted infections, versus a routine clinic-based urine collection in a one-to-one comparison study design: efficacy and acceptability among MSM in Belgium. BMJ Open, 2019. 9(4): p. e028145.
(8) Larsen, L.K., et al., DNA-Methylation-Based Detection of Urological Cancer in Urine: Overview of Biomarkers and Considerations on Biomarker Design, Source of DNA, and Detection Technologies. Int J Mol Sci, 2019. 20(11).
(9) Lin, S.Y., et al., Emerging Utility of Urinary Cell-free Nucleic Acid Biomarkers for Prostate, Bladder, and Renal Cancers. Eur Urol Focus, 2017. 3(2-3): p. 265-272.
(10) Fujita, K. and N. Nonomura, Urinary biomarkers of prostate cancer. Int J Urol, 2018. 25(9): p. 770-779.
(11) Lu, T. and J. Li, Clinical applications of urinary cell-free DNA in cancer: current insights and promising future. Am J Cancer Res, 2017. 7(11): p. 2318-2332.
(12) Merchant, M.L., et al., Isolation and characterization of urinary extracellular vesicles: implications for biomarker discovery. Nat Rev Nephrol, 2017. 13(12): p. 731-749.
(13) Salih, M., R. Zietse, and E.J. Hoorn, Urinary extracellular vesicles and the kidney: biomarkers and beyond. Am J Physiol Renal Physiol, 2014. 306(11): p. F1251-9.
(14) Bosschieter, J., et al., A protocol for urine collection and storage prior to DNA methylation analysis. PLoS One, 2018. 13(8): p. e0200906.
(15) Jung, M., et al., Changes in concentration of DNA in serum and plasma during storage of blood samples. Clin Chem, 2003. 49(6 Pt 1): p. 1028-9.
(16) Cheng, T.H.T., et al., Genomewide bisulfite sequencing reveals the origin and time-dependent fragmentation of urinary cfDNA. Clin Biochem, 2017. 50(9): p. 496-501.