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Stability of urinary cell-free DNA and detection of T790M variant

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Background

The so-called "liquid biopsy" has become a powerful tool for cancer research during the last decade, with circulating cell-free DNA (cfDNA) that originates from tumors as one of the most promising analytes. In contrast to plasma-derived cfDNA, only a few studies have investigated urinary cfDNA. One reason might be quick degradation and hence inadequate concentrations for downstream analysis. In this study, we examined the stability of cfDNA in urine using different ways of preservation under various storage conditions.

Methods

Synthetic cfDNA reference standard with T790M

To establish the experimental set up and to model clinical samples, we used the Multiplex I cfDNA Reference Standard Set in Synthetic Plasma (Horizon Discovery, Cambridge, UK), which contained DNA fragments with the size of 170 bp and covered eight onco-relevant mutations in the genes EGFR, KRAS, NRAS and PIK3CA with different allelic frequencies (AF) 0.1%, 1%, 5% and wildtype (wt). We focused on the T790M mutation on the epidermal growth factor receptor (EGFR) gene.

Collection and preparation of urine samples from healthy donors

For sample preparation, urine samples from four male and four female healthy volunteers were collected, each contributing a volume of 60-90 ml. Urine was pooled and experiments were set up using aliquots thereof.

Choice of urine preservation buffers for cfDNA stabilization

We decided to test three different buffers. Urinary Analyte Stabilizer (UAS, Novosanis, Wijnegem, Belgium) Urine Conditioning buffer (UCB, Zymo Research, Freiburg, Germany) and a buffer called "AlloU", which we prepared according to the published main components [1].

Experimental sample set up to mimic a patient sample during clinical routine

The setup included four different storage periods: 0 h, 4 h, 12 h and 24 h. They were supposed to represent the scenario that a clinical sample would be processed immediately, or after 4 h or, because it was forgotten, later during the day or even the next day. Further, the entire setup was applied to two different temperatures: room temperature (RT) and fridge temperature (4°C). The volume of the cfDNA reference standard solution was 60 µl for each spike-in, which equaled 24 ng of cfDNA in 3 ml of urine. For storage periods over 0 h, 4 h and 24 h the reference cfDNA standard with 5% AF was used, as these samples would be applied to subsequent mutation detection using ddPCR (**Table 1**).

Stabilizing reagent	Sample type	Urine volume	Buffer volume	Reference cfDNA spike-in volume
No buffer	NC	3 ml	-	-
No buffer	spike	3 ml	-	60 µl
U A S	NC	3 ml	1 ml	-
U A S	spike	3 ml	1 ml	60 µl
AlloU	NC	3 ml	120 µl	-
AlloU	spike	3 ml	120 µl	60 µl
UCB	NC	3 ml	210 µl	-
UCB	spike	3 ml	210 µl	60 µl

Table 1: Pipetting scheme of sample setup. The negative control (NC) contained the same buffer (or no buffer) as the corresponding sample containing cfDNA spike-in for a better comparison.

Extraction and quantification of ucfDNA

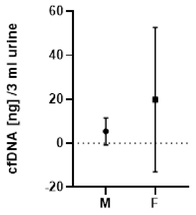
Isolation of cfDNA from model urine samples was performed using the QIAamp MinElute ccfDNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturers' instructions. For quantitation of cfDNA yield, we used the Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, USA). For ucfDNA quantification and detection of T790M variant we employed ddPCR using the QX200 Droplet Digital PCR System (Bio-Rad, California, USA) and the EGFR T790M assay (dHsaMDV2010019) (Bio-Rad, California, USA), according to the manufacturers' protocol. Based on that, fractional abundance (FA) of the mutant molecule in the wildtype DNA was calculated using QuantaSoft™ . For positive-template controls, cfDNA reference standard solutions were used with the 5%, 1% and 0.1% AF.

References: 1) Zhou, Qiang; Liu, Feng; Guo, Luoying; Chen, Ruoyang; Yuan, Xiaodong; Li, Chao et al. (2021): A novel urine cell-free DNA preservation solution and its application in kidney transplantation. In Nephrology (Carlton, Vic.) 26 (8), pp. 684–691.

Results

Analysis of ucfDNA in healthy urine samples

Eight different urine samples were analyzed individually before pooling. Sample donors were four female (F1-F4) and four male healthy persons (M1-M4). The cfDNA was extracted from the pure samples of 3 ml (triplicates), without using any spike-in and "natural" cfDNA concentration was measured fluorometrically. The individual cfDNA levels varied among the donors and ranged from not detectable (<0.1 ng) in M1 to a mean of 73.53 ng in F2. Less than 3 ng in 3 ml of urine were obtained in 5 out of 8 individuals. Apart from F2, M2 and M4 were also slightly elevated with 14.13 and 6.03 ng. There was no significant difference between ucfDNA levels in males and females (p=0.15). The mean value of all samples was 12.56 ng in 3 ml of urine (mean females: 19.75 ±31.44 ng; males: 5.37 ±5.85 ng; **Figure 1).**



Analysis of model urine samples spiked with cfDNA reference standard and matching negative controls

The different buffers elicited various cfDNA dynamics throughout the chosen storage conditions (**Figure 2**).

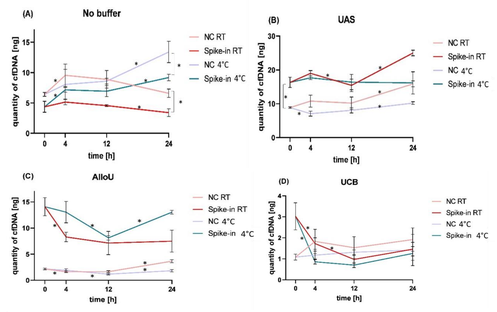


Figure 2: cfDNA dynamics in the NC and the samples spiked with cfDNA reference standard are depicted as mean values with their standard deviation for (A) no buffer, (B) buffer UAS, (C) buffer AlloU and (D) buffer UCB. The cfDNA was measured in ng after storage periods of 0 h, 4 h, 12 h and 24 h at room temperature (RT, red) and fridge temperature (4 °C, blue). The lighter colors show cfDNA levels of the NC. * indicates level of significance p≦0.05.

Relative cfDNA dynamics in spiked urine samples

The relative cfDNA dynamics after subtraction of the NC from each condition indicated that preservation with UAS and AlloU resulted in superior recovery of the spiked reference material compared to UCB or no buffer. At RT, the highest yields were achieved with UAS (**Figure 3A**) and at 4°C, AlloU resulted in the highest cfDNA levels after 24h (**Figure 3B**). Storage with UCB revealed a significantly lower initial relative cfDNA yield already at 0h compared to UAS (p=0.004) and AlluO (p=<0.001). The relative yield without any buffer was negative.

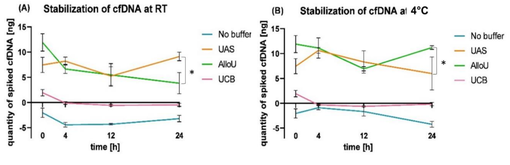


Figure 3: Comparison of relative cfDNA levels (ng) in four different preserving conditions after 0 h, 4 h, 12 h and 24 h at (A) room temperature (RT) and (B) 4 °C. The measured cfDNA levels in ng of the negative controls were subtracted from the cfDNA levels of the spiked samples. **After 24h at RT, UAS resulted in a superior relative cfDNA yield of 9.12 ng from 3 ml urine, which was significantly higher compared to 3.82 ng recovered with AllUo (p=0.015), but not significantly different from the initial level at 0h (p=0.169).** Further, using AlloU revealed significantly decreased cfDNA levels after 24 h at RT (p=0.007). After 24 h at 4 °C, however, UAS showed a relative cfDNA yield of only 4.28 ng which was a non-significant decrease compared to the initial level (p=0.248) ,but inferior to AlloU with 11.21 ng in 3ml urine after 24 h (p=0.034).

Observation of cfDNA dynamics in NC samples

We compared cfDNA dynamics in the NC samples (without synthetic reference cfDNA) under all four preserving conditions and at both temperatures (**Figure 4**).

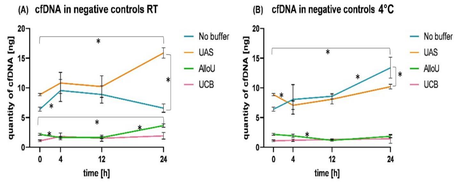


Figure 4: Comparison of cfDNA levels in the NC samples with the four conditions after 0 h, 4 h, 12 h and 24 h at **(A)** room temperature (RT) and **(B)** 4 °C.

Detecting the T790M variant in urinary cfDNA using ddPCR

The T790M mutation was measured in the samples preserved with UAS after 0 h, 4 h and 24 h storage at both temperatures. At RT, the FA was slightly lower compared to 4 °C, but overall the values were stable over time (FA (RT vs. 4°C): 0h: 2.05%, 4h: 2.0% vs. 3.2%, 24 h: 1.9% vs. 1.9%; **Figure 5**). Notably, the FA was around 2%, which was less than half of the spiked AF (5%). In the AlloU buffered samples, T790M was present after 0 h, 4h and 24 h storage at 4 °C. However, after 24 h at RT, the mutation was not detectable any more in samples with this buffer (FA (RT vs. 4°C): 0h: 2.95%, 4h: 2.25% vs. 2.5%, 24h: n.d. vs. 2.0%). At 4 °C, the values ranged between 2% and 3% FA. In samples preserved with UCB or no buffer, T790M was not detectable.

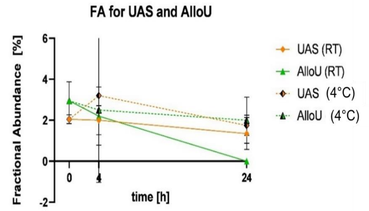


Figure 5: Fractional Abundance (FA) of T790M mutation in samples buffered with UAS and AlloU, respectively.

If cfDNA levels in the NC were high, like it was the case for the UAS buffered samples, the FA would turn out lower than 5%. The expected FA for UAS and AlloU preserved samples were calculated with the following equation:

$$FA = 5\% * (1 - \frac{NC}{Spike\text{-}in}).$$

The EGFR variant T790M in samples preserved with UAS had nearly the same FA measured with ddPCR compared to the calculated FA, which was slightly higher. The measured FA for the samples with buffer AlloU did not meet the calculated values as they were much lower (**Table 2**).

storage conditions		samples buffered with UAS		samples buffered with AlloU	
time [h]	temperature	Expected FA of variant [%]	Measured FA of variant [%]	Expected FA of variant [%]	Measured FA of variant [%]
0		2.41	2.05	4.26	2.95
4	RT	2.41	2.00	3.95	2.25
4	4°C	3.31	3.20	4.22	2.50
24	RT	1.96	1.90	2.65	0
24	4°C	1.25	1.80	4.39	2.00

Table 2: Expected FA versus measured FA of EGFR T790M for samples preserved with UAS and AlloU.

Conclusion & Outlook

In our study, the most effective stabilization of spiked cfDNA reference standard was achieved using the UAS buffer. However, the total spiked amount of 24 ng (in 3 ml urine) was not recovered in any of the experiments. We attribute this result to the limited extraction efficacy of the QIAamp MinElute ccfDNA Kit, which was approximately 50% for the cfDNA reference standard according to the manufacturers' product information. Our preliminary experiments confirmed that up to 50% of the spiked cfDNA amount could be recovered using the UAS buffer. Further, the positive control using distilled water instead of urine, to rule out nuclease activity, confirmed that only 55% of the reference standard could be recovered (after 0 h; data not shown). Taken together, using the UAS buffer resulted in stable cfDNA recovery over time and at both temperatures. We successfully showed that ddPCR was very specific for the T790M mutation. Furthermore, the majority of samples revealed similar cfDNA concentrations as measured fluorometrically in comparison to quantification by ddPCR. For the first time, we systematically compared different urine stabilizing buffers over time and at different temperatures. We were able to identify future challenges in the pursuit of standardization of ucfDNA collection and processing for downstream diagnostic procedures. We observed different ucfDNA levels under various conditions and found that **direct preservation with UAS showed the best results**, ensuring sufficient ucfDNA quality for downstream analysis such as the detection of a specific variant even after overnight storage at room temperature.