Enhanced ctDNA extraction with quaternary ammonium salt integrated in a ctKRAS prototype assay significantly improves sensitivity for detecting low-allelic frequency KRAS mutations in plasma

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Background

Liquid biopsy offers opportunities for the management of colorectal cancer, such as: diagnosis, therapy selection, monitoring and minimal residual disease assessment. Yet, routine adoption has been hindered by the need for high technical expertise, sample batching, and high cost. The Idylla™ platform is a fully automated sample-to-result, real-time PCR system that can overcome these obstacles. Research Use Only (RUO) Assay cartridges for plasma are available to genotype KRAS, NRAS, BRAF, and EGFR. A growing need for enhanced sensitivity triggered the development of a novel 4-ml circulating tumor DNA (ctDNA) extraction method based on quaternary ammonium salt. To evaluate the sensitivity of the novel ctDNA extraction technology, a 2nd generation Idylla™ ctKRAS prototype was developed including the quaternary ammonium salt based ctDNA extraction method.

2nd generation Idylla™ ctKRAS prototype

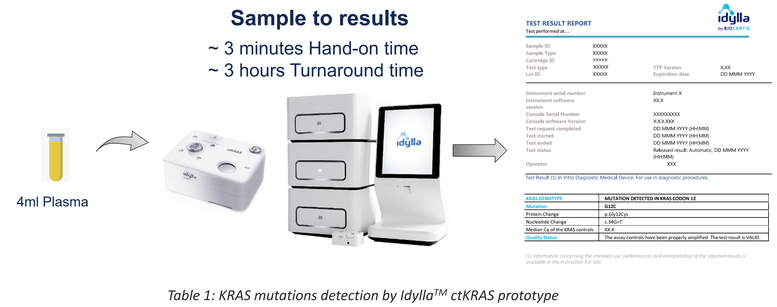

Table 1: KRAS mutations detection by Idylla™ ctKRAS prototype

Table 1: Advantages of the 2nd generation Idylla™ ctDNA prototype using a Quaternary ammonium-based extraction versus the 1st generation Idylla™ ctDNA assay using Guanidinium based extraction.

	1st generation Idylla™ ctKRAS: Guanidinium based extraction	2nd generation Idylla™ ctKRAS: Quaternary ammonium-based extraction
Error rate	Guanidinium salts are prone to precipitation increasing the risk for cartridge processing errors	Quaternary ammonium-based reagent demonstrated a lower viscosity profile resulting in a decreased error occurrence.
Input volume	Guanidinium salt saturation concentration is low, limiting the sample to buffer ratio for a given volume (i.e. maximum 1 ml of plasma can be processed in a cartridge).	Quaternary ammonium salts saturation concentration is high allowing higher sample to buffer ratio for a given volume (i.e. up to 4 ml of plasma in a cartridge). This increased input volume and allows to detect lower concentration of ctDNA.
Extraction yield	Suboptimal extraction yield compared to manual bench extraction method.	Around 80% extraction yield efficiency compared to manual bench extraction method;
Safety profile	High concentration of Guanidinium salt required for the extraction. Severely toxic, irritating, corrosive risk in case of leakage.	Low concentration of quaternary ammonium salt required for the extraction. Lower toxicity, irritability, and corrosive risk in case of leakage.
PCR inhibition	Sample preparation and extraction reagent carryovers present in up to 2% of the final reaction causing a less robust PCR performance.	Less carryovers of sample preparation and extraction reagents resulting a more robust PCR performance. Depends on the particular qPCR assay.

Samples and Methods

A proof-of-concept study was performed to evaluate the sensitivity of the novel ctDNA extraction technology, using KRAS G12C, G12D, and G12V.

The KRAS mutants (gBlocks of 170 bp) were spiked in a background of normal human plasma matrix and fragmented ctDNA (around 170 bp) (Acrometrix), mimicking the natural background of cell-free DNA in human plasma. The 4-ml samples were prepared at two input levels, a medium input level (10 ng ctDNA/ml) and a low input level (2.5 ng ctDNA/ml) representative for an average and a minimum concentration of ctDNA observed in patient plasma samples, respectively (Phallen *et al.*, 2017). For each input level and KRAS mutation six replicates of a 2-fold serial dilution were tested with the 2nd generation Idylla™ ctKRAS prototype to define the lowest level detected with a 100% hit rate. To demonstrate the improved performance compared to the 1st generation ctDNA extraction method, six replicates of a 2-fold serial dilution at medium input were also tested with the 1st generation Idylla™ ctKRAS Mutation Assay (RUO, not for use in diagnostic procedures).

The detectability of KRAS G12C was afterwards confirmed in human plasma samples spiked with 12.5 cps/ml G12C mutant (gBlock) and tested with the prototype.

RESULTS

The results of testing the KRAS mutants (gBlocks of 170 bp) spiked in a background of normal human plasma matrix and fragmented ctDNA (around 170 bp) (Acrometrix) with the 2nd generation Idylla™ ctKRAS prototype are presented in Figure 1 and Table 2.

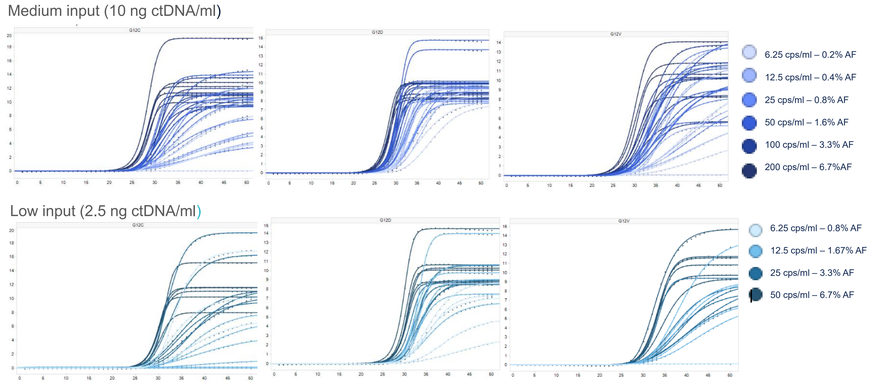

Figure 1: Amplification curves of G12C, G12D, and G12V spiked in a background of normal human plasma matrix and fragmented ctDNA.

Table 2: The lowest concentrations of mutations detectable with a 100% hit rate (6/6) in plasma using the Idylla™ ctKRAS prototype with the novel ctDNA extraction technology at low and medium input.

Target	Medium input (10 ng ctDNA/ml)		Low input (2.5 ng ctDNA/ml)	
G12C	0.4% AF	12.5 cps/ml	3.3% AF	25 cps/ml
G12D	0.2% AF	6.25 cps/ml	1.67% AF	12.5 cps/ml
G12V	0.4% AF	12.5 cps/ml	3.3% AF	25 cps/ml

Table 3: Comparison between the lowest concentrations of mutations detectable with a 100% hit rate (6/6) in plasma between the 1st and 2nd generation Idylla™ ctKRAS at medium input (10 ng ctDNA/ml).

Target	1st generation Idylla™ ctKRAS Mutation Assay (RUO)		2nd generation Idylla™ ctKRAS prototype	
G12C	0.8% AF	25 cps/ml	0.4% AF	12.5 cps/ml
G12D	3.3% AF	100 cps/ml	0.2% AF	6.25 cps/ml
G12V	0.8% AF	25 cps/ml	0.4% AF	12.5 cps/ml

The test results with the 2nd generation Idylla™ ctKRAS prototype show that 10-20 cps/ml can be successfully detected in both medium and low DNA background levels (Table 2). This is an improvement of 2-10 fold compared to the 1st generation Idylla™ ctKRAS Mutation Assay (RUO) (Table 3). This improvement is partially related to the higher plasma volume that can be used as input (4–fold increase in mutant cps in the PCR reaction). For some biomarkers, the improvement is higher than a 4-fold increase in sensitivity, which demonstrates that also the qPCR assay performance improves for these biomarkers when quaternary ammonium extracts are used compared to guanidinium extracts and that a lower % AF can be successfully detected. This indicates that the quaternary ammonium extracts contain a lower amount of PCR inhibitors, which can be extraction reagents (e.g. ethanol, guanidine) or sample derived inhibitors (e.g. anticoagulants or plasma proteins).

During the confirmation testing of G12C at 12.5 cps/ml in clinical specimen with the 2nd generation Idylla™ ctKRAS prototype, compatibility for both Streck and K2-EDTA tubes was demonstrated (Figure 2) .

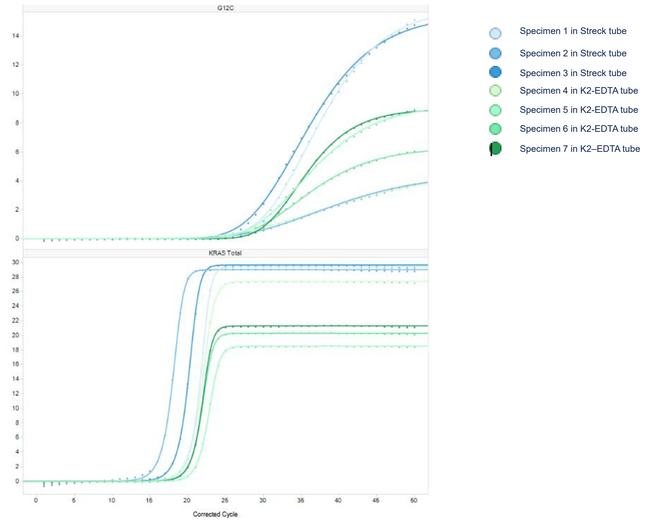

Figure 2: Amplification curves of G12C and KRAS control for testing 12.5 cps/ml G12C mutant in different human plasma samples.

All test results were available within 3 hours for all samples tested and the hands-on time was less than 5 minutes.

All 43 test runs were successful, no errors were observed. This demonstrates that this 2nd generation Idylla™ ct extraction technology is compatible with relatively high plasma volumes (4 ml) without causing clogging issues in a fully integrated system (sample in – results out).

All clinical human plasma samples spiked with 12.5 cps/ml G12C mutant (gblock), results in a G12C detection. This confirms that KRAS G12C can be detected at 0.4% AF at medium input (10 ng ctDNA/ml). This is in line with sensitivities obtained by FDA approved tests for KRAS G12C detection:

- Guardant 360 CDx with a sensitivity of 0.5% AF at high input (30 ng ctDNA) and 1.5% AF at low input (5 ng ctDNA) approved as CDx for LUMAKRAS™ (sotorasib).
- Agilent Resolution ctDx FIRST assay with a sensitivity of 0.58% AF at medium input (15 ng ctDNA) approved as CDx for KRAZATI™ (adagrasib).

Conclusion

The novel ctDNA extraction technology, based on quaternary ammonium salt, integrated in Idylla™ allows to develop fully automated sample-to-result prototypes able to reliably detect mutations in plasma as low as ±10-20 mutant copies/ml, which corresponds with 0.2-0.4% AF in an average patient plasma sample (at medium input). This is a clear improvement compared to guanidinium-based extraction technology.

References
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