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Development of a Liquid Biopsy Assay to Longitudinally Monitor Changes in Mutations' Allelic Frequency in Response to KB-0742

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Abstract

- Liquid biopsies analyzing cell-free DNA (cfDNA) released by tumors (ctDNA) are emerging as a powerful, noninvasive sampling approach. As opposed to single-site tumor biopsy, liquid biopsies allow for better representation of the tumor mutational landscape. Here we describe our approach to developing a liquid biopsy assay that will allow us to monitor, in real time, longitudinal changes in mutations' allelic frequency in patients. Our approach combines 2 complementary technologies: next-generation sequencing (NGS), which enables the identification of a broad array of mutations, and droplet digital polymerase chain reaction (ddPCR), which provides speed, sensitivity, and a custom, cost-effective advantage to monitor mutations in ctDNA obtained from patient plasma samples.
- We developed an approach that enabled us to meet the following criteria: 1) generation of a baseline and end-of-treatment (EoT) mutational profile for each patient using either a tumor tissue sample or liquid biopsy to identify both driver and potential resistance mutation markers, 2) longitudinal sampling and real-time analysis over the course of treatment, and 3) high assay sensitivity to detect low frequency alleles. Assay development included custom quantification of cfDNA for all patient samples. For individual mutations, gBlocks™ and/or cell-line–derived contrived cfDNA samples were used to optimize annealing temperature, time, cycle number, and input amount to attain an assay with a target 0.1% limit of detection (LoD) variant allele fraction (VAF). These customized mutation assays can potentially serve as an accelerated, cost-effective surrogate approach, compared to imaging, to monitor in real time the response to KB-0742 (a cyclin-dependent kinase 9 [CDK9] inhibitor) in the ongoing phase 1/2 clinical trial (NCT04718675).

Clinical Strategy of Using cfDNA Liquid Biopsies for Longitudinal Monitoring of Patient Response

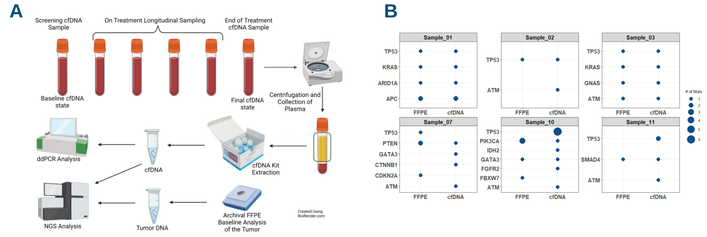
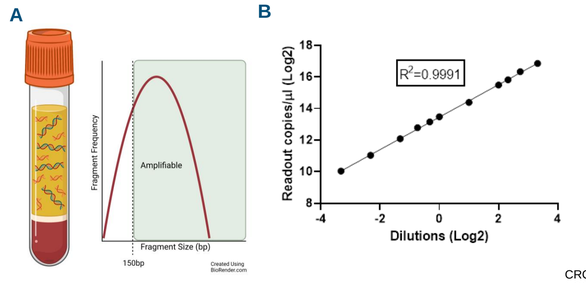


Figure 1: (A) In the KB-0742-1001 clinical trial, peripheral blood is collected from patients during screening, on day 15 of each treatment cycle, and at the EoT visit. Plasma is extracted using a double spin protocol within 72 hours of collection, and cfDNA is isolated using the Chemagen cfDNA extraction kit. A pretreatment formalin-fixed paraffin-embedded (FFPE) tumor sample is also obtained for each patient and analyzed using NGS. ddPCR assays are developed and validated based upon the mutation results of both tumor and baseline liquid biopsy results. **(B)** Pretreatment FFPE samples are typically archival samples; thus, depending on age of sample and patient treatment history, they may not accurately represent the current mutational profile of the tumor. To alleviate this concern, the FFPE NGS results were compared with screening cfDNA mutation profile. Strong correlation has been observed between the 2 sample types of patients analyzed thus far.

RPP30 ddPCR Reference Assay Provides a Highly Reproducible and Quantifiable Measurement of cfDNA Template Inputs



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Liquid biopsy of	Measured cfDNA copies/µl
CRC	141±3.5
NSCLC	17.75±0.04
NSCLC	2.45±0.2
Ovarian	10.05±0.1
Ovarian	5.55±0.2
Pancreatic	7.75±0.7
CRC	17.85±0.4
Breast	73.75±0.5
Breast	8.8±0.8
Breast	6.35±0.2
Breast	8.15±0.04
CRC	20.55±1.1
CRC	20.3±0.3

CRC = colorectal carcinoma;
NSCLC = non-small cell lung cancer.

Figure 2: (A) Isolated cfDNA from plasma consists of multiple fragment lengths. Only those fragments over 150 base pairs can be amplified in a ddPCR assay. In order to ensure consistency of input amounts across all samples, amplifiable DNA content needs to be determined. **(B)** An essential (housekeeper) gene, *RPP30*, was used to measure amplifiable cfDNA. The gene encodes the human ribonuclease P protein subunit P30 and is highly conserved and generally not impacted by copy number variations. The *RPP30* assay was validated for accurate and quantifiable measurement of amplifiable cfDNA. Using gBlocks, *RPP30* ddPCR assay was performed across an input dilution range of 500 to 50,000 copies per 20 µl reaction. The *RPP30* assay readout was highly sensitive and correlated with the gBlock input across the entire dilution range. **(C)** *RPP30* assay was used to determine amplifiable copies in commercially sourced patient samples. The assay was able to detect cfDNA in all the samples tested across a broad range of concentrations. The readouts were highly reproducible as illustrated by the low observed variance between replicates.

Annealing Temperature Optimization for *TP53* p.M237K and *TP53* p.R280K ddPCR Mutation Detection Assays

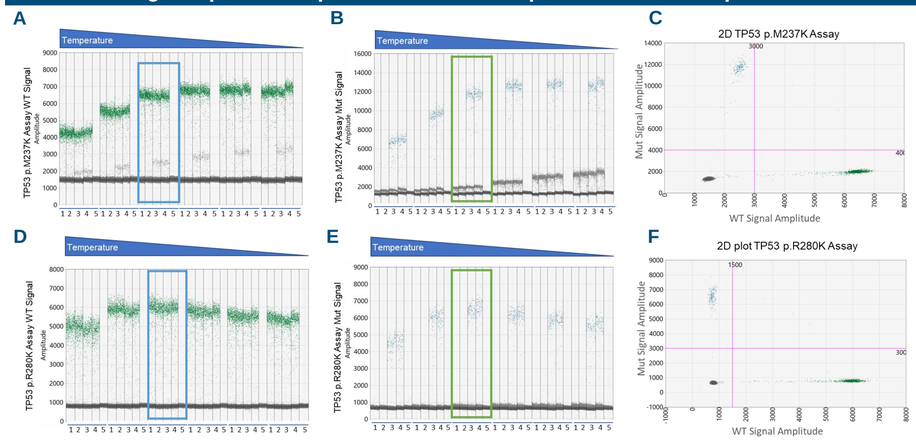

Figure 3: Two *TP53* mutations (p.M237K and p.R280K) were identified in patient samples from our clinical cohort. For both mutations, annealing temperatures for the probes were optimized using a temperature gradient of 50°C to 58°C. The reactions run in this assay contained the following input materials in replicates: (1 and 2) Wild-type (WT)–only gBlocks, (3 and 4) WT and 16% mutant (Mut) gBlocks, and (5) no template control (NTC). *TP53* p.M237K 1-dimensional (1D) plots of the WT signal amplification **(A)** and Mut signal amplification **(B)** levels indicated an optimal annealing temperature of 54°C for both set of probes (*blue and green boxes*) due to reduction in rain and distance from background signal. The 1D plots for *TP53* p.R280K are shown in **(D)** for WT signal amplification and **(E)** for Mut signal amplification. Using the same metrics as before, the optimal annealing temperature was 54°C as well for both set of probes (*blue and green boxes*). The 2-dimensional (2D) plots for *TP53* p.M237K **(C)** and *TP53* p.R280K **(F)** assays at the optimal annealing temperature (54°C) showing ideal separation between the 4 droplet population types (negatives, single positives, and double positives).

Specificity Test Demonstrates No Cross-reactivity With WT and Closely Positioned Mutations in *TP53*

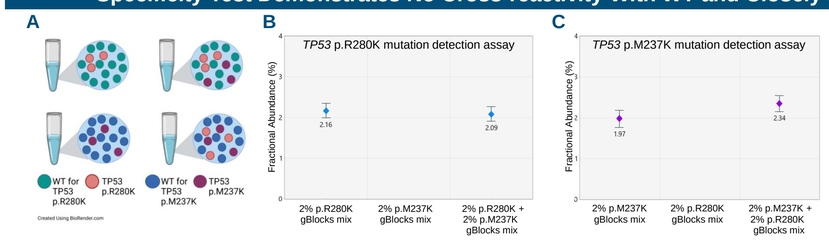

Figure 4: (A) Experimental design. Four different reactions were set up: 1) 2% p.R280K Mut in a background of WT gBlocks, 2) 2% p.M237K Mut in a background of WT gBlocks, 3) 2% p.R280K Mut and 2% p.M237K Mut in a background of WT gBlocks, and 4) 2% p.M237K Mut and 2% p.R280K in a background of WT gBlocks. To determine the specificity of each assay reactions 1, 2, and 3 were amplified using the *TP53* p.R280K assay probes **(B)** and reactions 1, 2, and 4 were amplified using the *TP53* p.M237K assay probes **(C)**. In both experiments, only the mutations specific to the probes were detected, indicating ideal specificity with no cross-reactivity to either the WT or the alternate Mut template.

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TP53 p.R280K ddPCR Mutation Detection Assay Has a Limit of Blank (LoB) at 0.04%

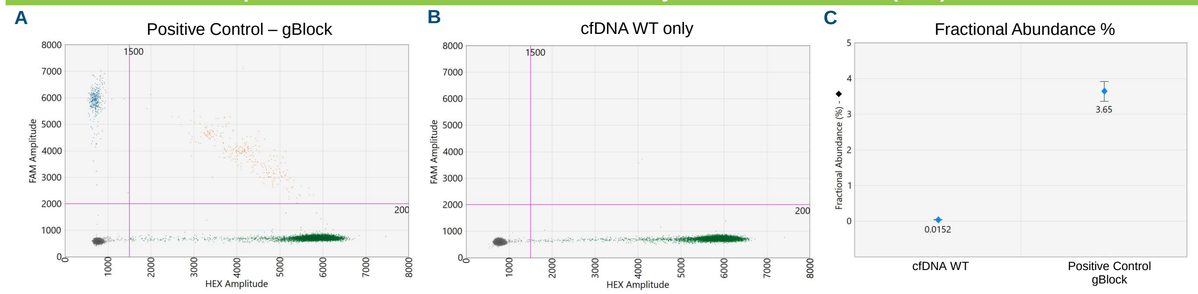

Figure 5: The LoB represents the fraction of false-positive droplets detected when a sample does not contain the target. To determine the LoB, a mix of Mut *TP53* p.R280K and corresponding WT gBlocks were used as a positive control, and 5 experimental reactions of WT-only cell-line–derived cfDNA (4,000 total copies per 20 µl reaction) were run and combined in the analysis. Using cfDNA rather than gBlocks to measure LoB better represents the complexity of patient samples. 2D plots of the reaction readouts for *TP53* p.R280K are shown in **(A-B)**. The thresholds in the 2D plots were set by determining the ideal separation between the 4 droplet population types (negatives, single positives, and double positives). The fractional abundance of *TP53* p.R280K for both gBlocks and cfDNA are shown in **(C)**. Average false-positive rate across the cfDNA WT samples is 0.015, with a 95% CI of 0.038, determining the LoB for this assay at 0.04%.

TP53 p.R280K ddPCR Mutation Detection Assay Has a LoD at 0.12%

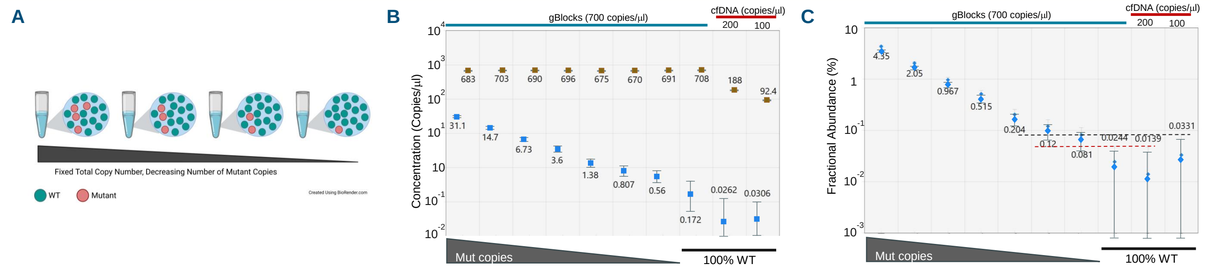

Figure 6: The LoD represents the lowest fractional abundance that we can detect with a 95% CI above the LoB. **(A)** used a fixed WT gBlock copy number with different dilutions of Mut gBlocks ranging from 100% to 0.06%. For comparison, cfDNA at 200 and 100 copies/µl were used to determine the consistency of the LoD across the 2 DNA input types. ddPCR was performed to determine how low a Mut prevalence can be detected with a 95% CI in a WT-only background. Amplifiable copy-number concentration across reactions (*brown=WT; blue=Mut*) are plotted in **(B)**. Fractional abundance of *TP53* p.R280K are shown in **(C)**. Based on the 95% CI, the LoD is 0.12% for the gBlocks input of 14,000 copies per 20 µl reaction (700 copies/µl), and for cfDNA input at 4,000 copies per 20 µl reaction (200 copies/µl). The dashed line represents 95% CI thresholds (*red for gBlocks and 200 copies/µl cfDNA; black for 100 copies/µl cfDNA*). Numbers on the plots indicate average fractional abundance, and error bars represent 95% CI.

TP53 p.R280K ddPCR Mutation Detection Assay Has a Limit of Quantification (LoQ) at 0.16%

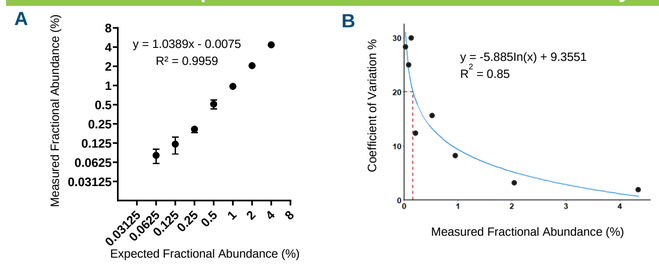

Figure 7: The LoQ represents the lowest allelic fraction that is both quantitative and accurate within a predetermined variance limit. To determine the LoQ, expected and measured fractional abundance were plotted and examined for assay accuracy. Data show high linear correlation $R^2 = 0.9959$ **(A)**. Percent coefficient of variation (CV) was determined for each fractional abundance and a regression model was fit to the data. For a precision goal of CV ≤ 20%, the LoQ was calculated as 0.16% allelic fraction **(B)**.

ddPCR Assay for *TP53* p.R280K Is Validated and Ready to Be Tested on Clinical Samples

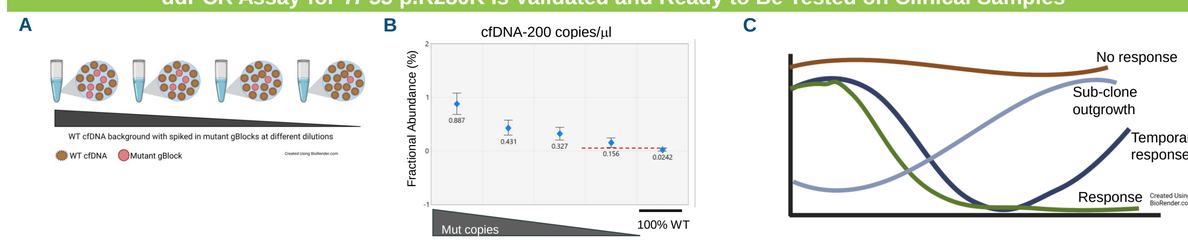

Figure 8: (A) As a proof of concept for the final validated assay, Mut gBlocks were spiked-in at different dilutions into WT cfDNA at 4,000 copies per 20 µl reaction (200 copies/µl). ddPCR was performed to confirm the optimized assay parameters determined using the gBlocks in the previous experiments. **(B)** Fractional abundance of *TP53* p.R280K LoD is at 0.16%, consistent with what was observed with the gBlocks. The dashed line represents 95% CI threshold. Based upon this experiment and the calculated LoB, LoD, and LoQ values with the gBlocks, we can confidently call VAFs of 0.16% and above. **(C)** Potential longitudinal monitoring outcomes include no reductions observed in VAF (*brown line*), early reduction followed by an increase in expression (*dark blue line*), continued reduction throughout (*green line*), or a subclone outgrowth (*light blue line*).

Conclusions

- Having a liquid biopsy collection and analysis plan allows for a real-time analysis of mutations detected at screening and end of treatment, indicating potential early antitumor activity and/or elucidating resistance mechanisms.
- *RPP30* quantification is an accurate and sensitive approach for cfDNA input normalization; a critical factor required for a robust, rare-mutation detection assay.
- The *TP53* p.R280K assay development and validation presented here represents our capability and strategy for subsequent custom validations of patient-specific mutations on the ddPCR platform in order to test longitudinal allelic frequency of mutations of interest from the ongoing clinical trial.

A phase 1/2 clinical trial of KB-0742 (NCT04718675) is currently recruiting patients with relapsed or refractory solid tumors or non-Hodgkin lymphoma.

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