Analytical Validation of the Signatera™ RUO Assay, a Highly Sensitive Patient-Specific Multiplex PCR NGS-Based Non-Invasive Cancer Recurrence Detection and Therapy Monitoring Assay

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Introduction

- The identification of tumor mutations in circulating cell-free DNA (cfDNA) holds great potential for the non-invasive detection of cancer relapse before clinical manifestation, detection of minimal residual disease after curative-intent treatment, and detection of therapeutically relevant mutations.
- Previously, we demonstrated 93% sensitivity, with no false positives, for early relapse detection in patients with early stage lung cancer using a proof-of-concept version of the Signatera™ assay.[1]

Objective

- To review and report analytical validation results for the detection of tumor-specific variants by the current version of the assay

Methods

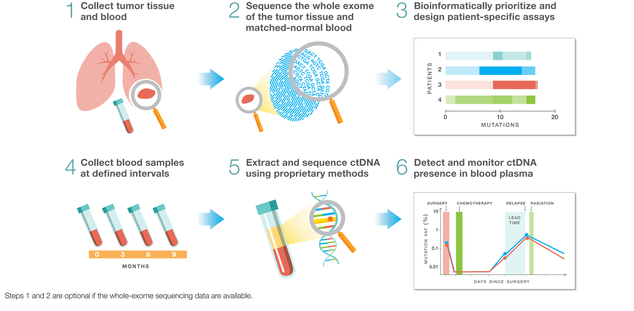
Signatera RUO

- The Signatera (RUO) process starts with identifying and prioritizing somatic mutations from whole-exome sequencing of tumor and matched normal samples. Patient-specific multiplex-PCR assays targeting 16 somatic single-nucleotide and indel variants are then assayed by massively parallel sequencing in plasma samples collected throughout the patient's disease course to help detect and monitor circulating tumor DNA (**Figure 1**).

Analytical Validation

- The analytical validation for the current version of the Signatera (RUO) assay was performed on two breast cancer cell lines (HCC2218, HCC1395), one lung cancer cell line (NCI-H1395) and their matched normal counterparts (HCC2218-BL, HCC1395-BL, and NCI-H1395-BL, respectively). Varying amounts of tumor cell line DNA (0%, 0.005%, 0.01%, 0.03%, 0.05%, 0.1%, 0.3%, 0.5%, 1%) were titrated into its respective matched normal cell line DNA.
- Multiplex-PCR assay primer pools (each consisting of 16 primer-pair assays specific to high-confidence somatic mutations) were designed using whole exome data from the corresponding tumor cell line DNA and its matched normal cell line DNA.
- The starting total input into library prep for each reaction was 20K genome equivalents; SNV and indel targets from the corresponding tumor DNA spike-in samples were amplified using the multiplex-PCR assay primer pools mentioned above.
- The mPCR products were barcoded, then pooled with other mPCR barcoded products, and subsequently sequenced on an Illumina HiSeq 2500 Rapid Run with 50 cycles of paired-end reads using the Illumina Paired End v2 kit with an average read depth of ~100,000/assay.

Figure 1. Signatera (RUO) Process

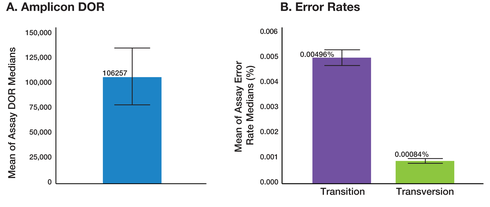


Steps 1 and 2 are optional if the whole-exome sequencing data are available.

Results

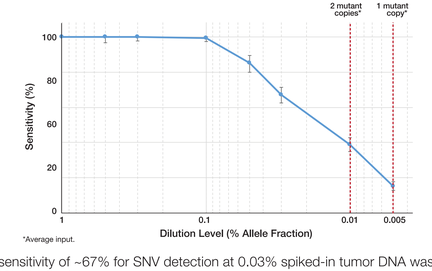
- **Figure 2** depicts sequencing QC process checks across 4 HiSeq PE 2x50 runs, including, background transition and transversion error rates, and average per target depth-of-reads (DOR) of ~100K (where any targets receiving less than 5K reads were considered failed and not taken into account for calling).

Figure 2. Plasma Sequencing QC



Each error bar is constructed using 1 standard deviation from the mean. DOR, depth of read.

Figure 3. Sensitivity of Single SNV Detection



*Average input.

- An analytical sensitivity of ~67% for SNV detection at 0.03% spiked-in tumor DNA was achieved with Signatera (RUO) (**Table 1**).

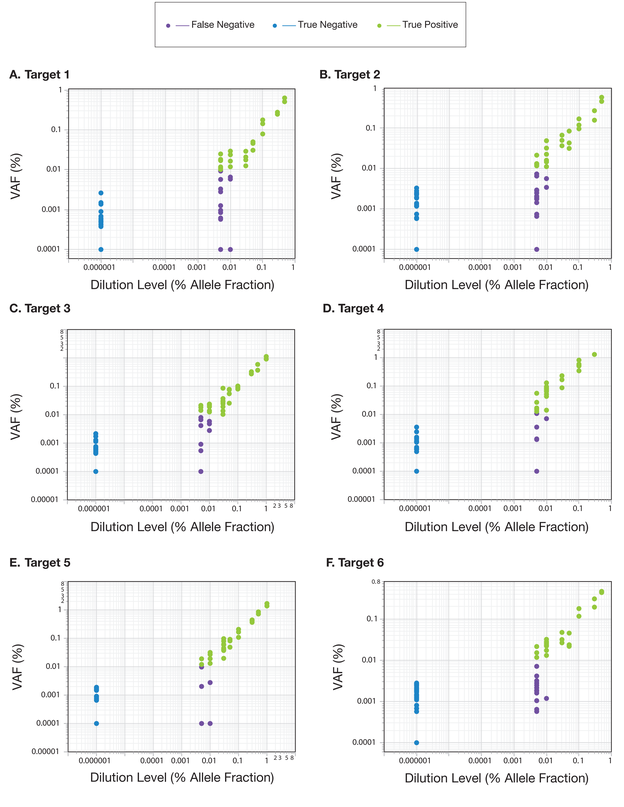
Table 1. Analytical Sensitivity Results for Signatera (RUO)

Tumor DNA Concentration (%)	Average Mutant Copies*	Detected SNVs	Total SNVs Used	Sensitivity (%)	95% CI
0.005	1	120	794	15.1	12.7 - 17.8
0.010	2	246	635	38.7	34.9 - 42.7
0.030	6	291	433	67.2	62.6 - 71.6
0.050	10	208	244	85.3	80.2 - 89.5
0.100	20	227	228	99.6	97.6 - 100
0.300	60	177	177	100	98 - 100
0.500	100	120	120	100	97 - 100
1.000	200	76	76	100	93 - 100

*Based on an average number of 20K starting genome copies, as expected from 10 ml of plasma.
An assay specificity of 99.9% for normal (0% spike-in) cell line DNA was achieved (Table 1, Figure 3).
SNV, single nucleotide variant.

- For a given set of SNVs, the number of expected input percentages versus detected variant allele frequency (VAF) percentages for the differing spiked-in mutant DNA concentrations is shown across six targets, displaying high sensitivity above 0.03% tumor DNA concentrations (**Figure 4**).
- The false negatives shown at ≤0.01% SNV input (starting with <2 mutant copies) include mutant molecules lost due to sampling (**Figure 4**).

Figure 4. Expected Input vs Observed VAF with Signatera (RUO)



- Estimated sample-level sensitivities for Signatera (RUO) when at least two SNVs are called from a set of 16 high-confidence SNVs, are listed in **Table 2**.

Table 2. Estimated Sample-Level Sensitivity

Tumor DNA Concentration (%)	Sensitivity (%)
0.005	49.97 -72.02
0.010	94.55 - 99.56
0.030	99.97 - 99.99
0.050	100
0.100	100
0.300	100
0.500	100
1.000	100

Conclusions

- The Signatera RUO assay provides a novel method of non-invasively detecting recurrence of personalized cancer signatures in plasma by ultra-deep sequencing of custom-made multiplex PCR assays (selected from a patient's tumor) up to 14.7 months in colorectal cancer (see Abstract Number 1590) with high sensitivity, high specificity, and low error rates.
- Based on the analytical validation results, the Signatera RUO assay, on an SNV-level, has a 99.9% specificity and a greater than 65% sensitivity above 0.03% tumor fraction and a 100% sensitivity above 0.1% tumor fraction.
- On a sample-level, the Signatera RUO assay has greater than 95% sensitivity at 0.01% tumor fraction, nearly 100% sensitivity at 0.03% tumor fraction, and 100% sensitivity at 0.05% and above tumor fraction.
- These data demonstrate high rate of detection at single molecule mutant levels; they also suggest that lower plasma volumes may be utilized to achieve the same single-molecule detection with high specificity.
- The performance of the Signatera assay suggests the potential for it to determine chemotherapy treatment effectiveness.

Reference

1. Abbosh C, et al. Nature. 2017. 545:446–51.

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