natera | Signatera™
Residual disease test (MRD)

A personalized, tumor-informed approach to detect molecular residual disease with high sensitivity and specificity

Design and analytical validation of Signatera™, the first ctDNA assay custom-built for detecting MRD and assessing treatment response

Introduction

Non-invasive monitoring of circulating tumor cells and molecular alterations has been an established method of detecting minimal residual disease in hematologic malignancies.[1,2] However, monitoring based on circulating-tumor DNA (ctDNA) for solid tumors has been limited by extremely low concentrations of ctDNA molecules, heterogeneity of tumor mutations among patients, and errors from high biological background noise or background noise from the assay itself.[3,4]

Signatera™ is the first patient-specific, custom built ctDNA assay for detecting molecular residual disease, and monitoring treatment response or recurrence. Signatera is unique in its ability to detect ctDNA at a variant allele frequency (VAF) of <0.1% of cell free DNA (cfDNA) from plasma.[5-9] This whitepaper summarizes advantages of the Signatera approach, data from the analytical validation of Signatera, and differences between Signatera and other ctDNA detection assays.

Biological principle of clonality

Clonal variants occur early in tumor evolution and remain present in every cancer cell as the tumor evolves. Most clonal variants are passenger mutations (not driver mutations). These mutations have no effect on the fitness of a clone but may be associated with a clonal expansion because it occurs in the same genome with a driver mutation. These clonal variants are unique to each individual tumor. Identifying and tracking a subset of clonal variants provides an opportunity to detect residual disease with high sensitivity and specificity, irrespective of tumor heterogeneity.

The Signatera approach

For the Signatera approach, somatic, clonal variants are identified by whole exome sequencing of the primary tumor and the matched normal (whole blood) sample. Following this, a bespoke assay of 16 tumor-specific, clonal, somatic variants are generated for each patient. The resulting "tumor signature," individualized to each patient's tumor, is monitored throughout the patient's disease course to detect the presence of tumor DNA in the plasma.[6-9]

There are several advantages with the Signatera approach. Analytical sensitivity and specificity of Signatera is enhanced due to improved library preparation and molecular recovery, significantly reduced PCR error, and advance knowledge of specific variants present in a patient's tumor. Furthermore, focusing on patient-specific variants enables ultra-deep sequencing (100,000X average depth of coverage) of each target to obtain a high level of confidence for a positive-ctDNA call, effectively lowering the limit of detection into the single–molecule range. The limit of detection for Signatera, measured in VAF, is 0.01%. This is equivalent to one mutant haploid genome in a background of 10,000 normal haploid genomes. Signatera is optimized to achieve high analytical specificity of >99.5%.[7-9] Combining a low limit of detection and advanced knowledge of clonal, tumor-specific variants is how Signatera achieves high sensitivity and specificity in ctDNA detection.[6]

Currently available assays for detecting ctDNA from patient plasma tests are typically static, tumor-naive panels that target hotspot or actionable mutations. Given the heterogeneity of cancer, even large static panels targeting up to more than a hundred of genomic loci might detect only a few mutations from a given individual's primary tumor.[11-13] Moreover, mutations identified in these panels may not be tumor derived, making such approaches less specific.[11-13]

The targeted mutations identified by these static panels may be driver or subclonal mutations which may be susceptible to treatment. Treatment may lead to selective attrition of cancer cells containing the susceptible mutations, thereby reducing the ability of those mutations to track the overall cancer burden. Additionally, the plasma-level VAF limit of detection (LOD) for these static panels starts at approximately 0.1%–1%, which is 10–fold lower than the 0.01% VAF LOD achieved with Signatera.[14-18]

Steps in the Signatera process

To monitor for cancer recurrence or to detect residual disease with ctDNA, the Signatera process starts with whole exome sequencing of the tumor tissue and the buffy coat from matched normal whole blood for each patient. Based on sequencing results, a list of somatic single-nucleotide variants (SNVs) specific to each patient and are present in the tumor but absent in the germline, are bioinformatically identified. Next, a proprietary algorithm is used to select a set of 16 somatic SNVs for multiplex PCR primer design based on several factors, including the clonality, detectability of tumor DNA if present in the plasma, frequency of the variants identified in the tumor tissue DNA, and background noise profile in the plasma. Cell free DNA libraries are prepared from each longitudinal blood sample, followed by patient-specific, 16-plex PCR. The amplicon products are tagged with sequencing barcodes and pooled for ultra-deep next generation sequencing, followed by data analysis to detect the presence or absence of ctDNA.[6]

Steps in the Signatera work flow are outlined below (figure 1):

Step 1: Primary tumor tissue and matched normal blood are collected from each patient. Genomic DNA from tumor tissue and buffy coat are extracted, whole-exome sequenced, analyzed, and filtered for patient-specific somatic mutations.

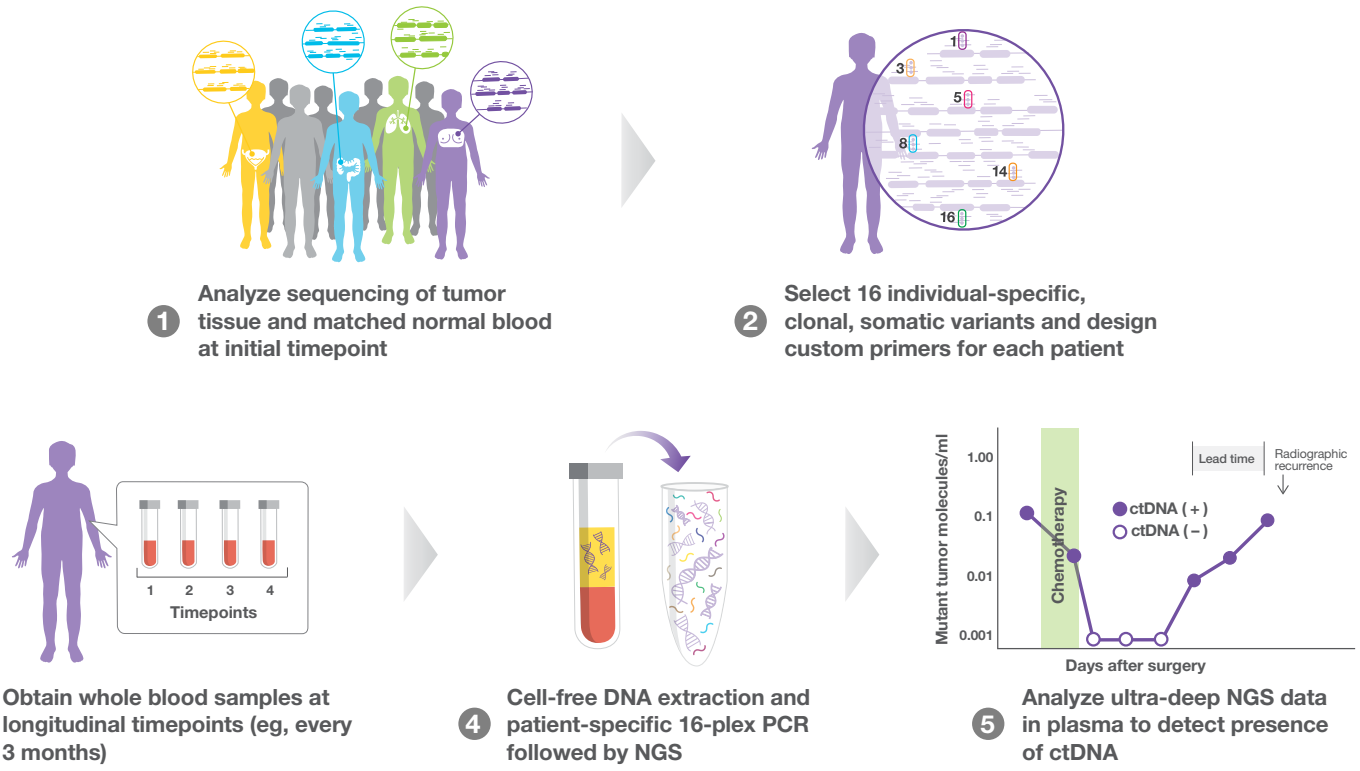
Step 2: The top 16 somatic variants are selected based on clonality, detectability, and frequency of mutations. Multiple-PCR compatible primers are designed for each of the 16 somatic variants.

Step 3: Whole blood is collected at predefined time points for longitudinal surveillance.

Step 4: Plasma are isolated and cfDNA extracted, followed by assaying with the patient-specific 16-plex PCR pool.

Step 5: Following multiplex PCR amplification, ultra-deep sequencing is performed. Next-generation sequencing data are analyzed to detect the presence of ctDNA.

Figure 1: Signatera workflow



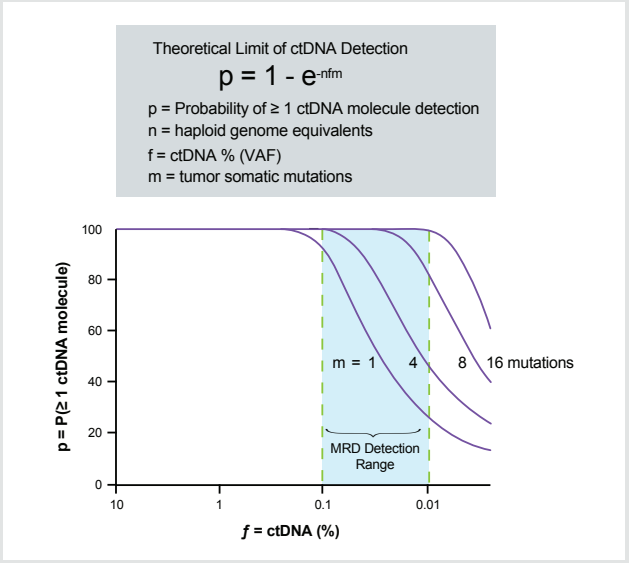
Rationale for targeting 16 variants with a personalized approach

The clinical sensitivity of MRD detection (range: 0.01%-0.1% VAF) is dependent on the number of somatic SNVs tracked. As represented in Figure 2, MRD detection of ≤0.1% VAF is compromised when targeting ≤8 clonal mutations. Based on this statistical model, more than 8 targets would be required to reliably detect ctDNA <0.1% VAF, which is critical for MRD detection immediately after surgery.

There are technically two ways to target >8 mutations:

1. Build a very large static liquid biopsy panel that can reliably cover at least 8 mutations in every single patient. Given the high inter-patient heterogeneity in cancer, this would require a very large panel that is economically unfeasible to implement in clinical practice
2. Introduce a personalized approach that only tests for the tumor-derived mutations in each patient. This is what we have done with Signatera

Figure 2: Clinical sensitivity of MRD detection



The Signatera assay amplifies cfDNA at 16 loci with custom-designed PCR primers targeting 16 clonal passenger mutations from the patient's tumor. We chose to target 16 because this allows for some redundancy, to confidently end up with at least 8 assays that are clonal and successful. By targeting 16 mutations, Signatera can confidently detect VAF down to 0.01%, equivalent to one mutant copy in a background of 10,000 genomic copies. In addition, the assay is optimized to achieve high sample level specificity (>99.5%) by requiring detection of at least two mutations for a ctDNA-positive call, leading to fewer false-positives. Table 1 represents the performance of Signatera compared to other commercially available static, liquid-biopsy panels commercially available.

Table 1: Average coverage of mutations from commercially available static panels versus Signatera

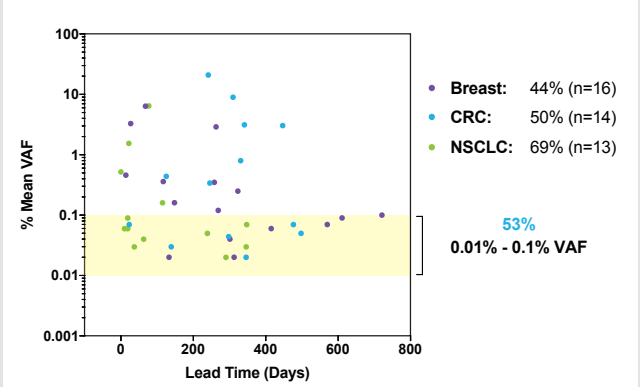
	Guardant360 (73 genes)	Roche Avenio (197 genes)	Signatera (16 SNVs)
Number of somatic variants per patient			
NSCLC (n=100)	3*,19	3†,22	16
Breast (n=49)	3*,20	3.7†,‡	16
Colorectal (n=130)	3*,21	15.7†,‡	16
Technical Specifications			
Genome coverage	75 kb	203 kb	3 kb
NGS coverage	8,000x	10,000x	100,000x

*Median †Average
‡Natera evaluated in silico the overlap in coverage between WES-derived mutational signatures and commercially available ctDNA assays. Note that these performance estimates assume 100% mutation detection in covered genes, which may not occur in practice depending on VAF, input quantities, base-level sensitivity, etc.

Clinical sensitivity of <0.1% VAF improves recurrence detection lead time

Among the 43 total cancer relapse cases (lung, colorectal, and breast cancers) analyzed at Natera, 53% (23/43) of the relapses had ctDNA first detected at VAF of 0.01%-0.1%.5,7,9 The lead time in ctDNA detection before clinical recurrence in patients with 0.01%-0.1% VAF was 264 days, which was 77 days ahead of the 187-day lead time for patients with ctDNA detected at >0.1% (Figure 3). This underscores the importance of a ctDNA monitoring test that can confidently detect MRD in the range of 0.01%-0.1% VAF, at the lowest level of residual disease burden, which hypothetically is when a patient has a greater chance to benefit from potentially curative treatment.

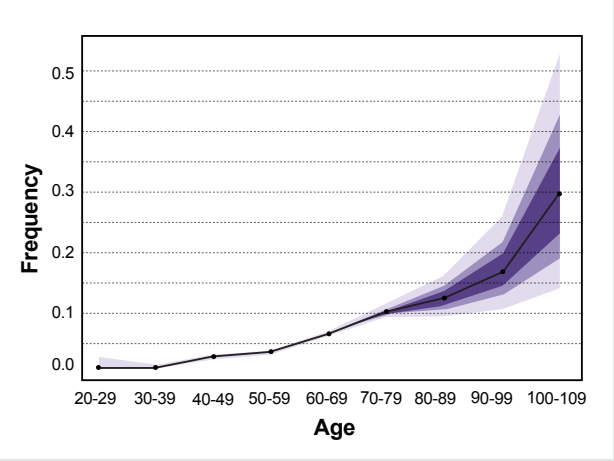
Figure 3: Percent VAF at the first time point when ctDNA was detected in patients with clinical relapse



Importance of sequencing DNA from both tumor tissue and normal cells

Clonal hematopoiesis of indeterminate potential (CHIP) is an age-related phenomenon where somatic mutations accumulate in cells of the blood or bone marrow.[23,24] CHIP mutations are a source of biological noise that are a common cause for false-positives in cfDNA analyses.[24] CHIP mutations are pervasive and are present in up to 20% of individuals (Figure 4).[23] Cell free DNA from CHIP mutations are almost indistinguishable from cfDNA of tumor derived mutations. The Signatera approach filters out CHIP mutations from the tumor tissue DNA using sequencing data from normal cells, thereby reducing false-positive results and focuses the ultra-deep NGS sequencing on a limited number of tumor-specific mutations per patient. Ensuring only tumor-specific variants are tracked is how Signatera achieves a low limit of detection (LoD) in the single–molecule range, at VAF 0.01%, which is 10-fold lower than that of conventional technologies (VAF 0.1%-1%).[14-18]

Figure 4: Prevalence of CHIP mutations, according to age



Designed without the need for molecular barcodes

Assays for low quantities of ctDNA detection often use molecular barcoding followed by hybrid capture as an approach to decrease error rates caused by process and sequencing-related artifacts. Molecular barcoding, also known as unique identifiers (UIDs), enable tagging and tracking of individual DNA molecules to distinguish somatic mutations from artifact mutations generat-ed during the PCR and sequencing process. During research and development of Signatera, molecular barcoding approaches were also explored.

However, use of molecular barcodes was found to sacrifice sensitivity without improving specificity, ultimately considered unnecessary, and not incorporated into the Signatera methodology.

There are several reasons why sensitivity and specificity may be compromised by approaches that utilize molecular barcoding, including hybrid capture and one-sided PCR:

- The depth and uniformity of sequencing is poor with hybrid capture, which decreases the quality of data across target sets. Specificity can also be variable across targets with non-uniform depth of read. At a minimum, a 5X to 10X sequencing depth per target input molecule is required to distinguish errors from mutations in the original target
- Hybrid capture has been reported to cause DNA oxidative damage, such as 8-oxoguanine and cytosine deamination, which could lead to false positive results[25-27]
- We have observed the formation of chimeric molecules from the hybrid capture process, which can appear as an original target molecule and contribute to false-positive calls. In principle, chimeric molecules should also occur with 1-sided PCR approaches and lead to false-positive calls
- The use of molecular barcodes is not robust enough for error correction with respect to input mass in hybrid capture and one-sided PCR approaches. In cases where there are low concentrations of ctDNA and more input mass is required, specificity will suffer

Due to the reasons above, Natera has developed other methods in the workflow to optimize the sensitivity and specificity of Signatera without use of molecular barcoding. Recently published analysis of tumor-naïve approaches by another group have shown high false-positive results despite use of molecular barcodes (Figure 5, Table 2).[28]

Figure 5: Variant concordance plot of plasma-testing by NGS from four different vendors using tumor-naïve approaches with molecular barcodes

Table 2: Sensitivity and positive predictive value of all variants with each vendor

Vendor	TP	FP	FN	Sensitivity* (%)	PPV† (%)
A	6	5	10	38	55
B	8	2	3	73	80
C	17	10	2	89	63
D	13	23	6	68	36

Abbreviations: FN, false negative; FP, false positive; PPV, positive predictive value; TP, true positive

*Sensitivity was calculated by dividing TP calls by the sum of TP and FN calls.
†Positive predictive value was calculated by dividing TP calls by the sum of TP and FP calls

Signatera prototype in the TRACERx study

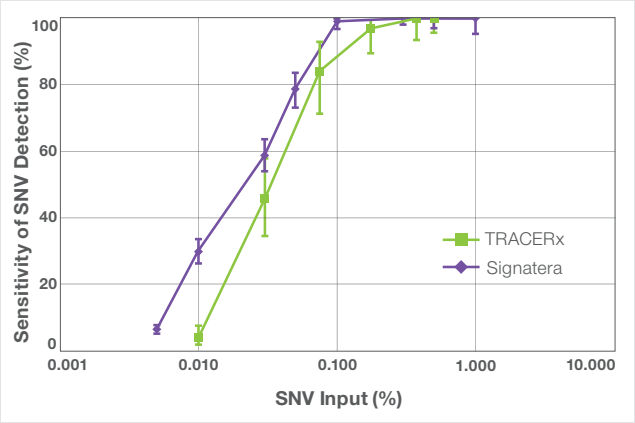
Proof-of-concept of Signatera was first established in the NSCLC, TRACERx study using a Signatera prototype.[5] Multi-region exome sequencing of tumor samples from stage I-III NSCLC patients was performed to construct phylogenetic trees. Multiplex PCR primers were designed to detect clonal and subclonal variants through ctDNA analysis. Subsequent multiplex PCR sequencing on plasma samples from 24 patients was performed. Molecular relapse was observed in 13/14 relapsed patients, up to 346 days earlier than standard radiological confirmed relapse (average lead time of 70 days) by both clonal and subclonal selected SNVs.[5] A sensitivity of 93%, with no false positives, was demonstrated for early relapse detection in patients with stages I–III lung cancer.[5] When results produced in the TRACERx study for the same lung cancer cohort were compared with a generic lung panel, TRACERx identified 10 out of 10 ctDNA positive early-stage lung cancer cases compared to 7 out of 10 cases detected using a static, hotspot lung panel.[5]

Following the TRACERx study publication, multiple improvements have been made to produce a more automated and scalable work flow with higher molecular recovery and lower error, which yielded 5 times improved analytical sensitivity. The analytical validation for the assay in TRACERx was performed using synthetic SNV spikes and mixing libraries directly into the multiplex PCR test, which does not account for molecular loss during library preparation or differing DNA fragmentation. In contrast, the analytical validation for Signatera started with cfDNA mixtures from the tumor and matched normal samples during the library prep process. A comparison of the analytical sensitivity between the prototype and Signatera, as shown in Figure 6, demonstrates the sensitivity improvements that have been made since the TRACERx study at tumor DNA concentration levels below 0.5%.

The key improvements with Signatera since the TRACERx study are detailed below:

- At the single SNV level, detection at 0.01% VAF improved from <5% to 30%
- At a sample level, this implies detection improvement at 0.01% VAF from <15% to 85-97%
- Drivers of improvement:
  - Increased average depth of read from 40,000x to 100,000x
  - Improved variant calling algorithm
  - Optimized chemistry and workflow to reduce errors
  - Improved sample prep efficiencies (library prep and cfDNA extraction)
  - Selection of more clonal variants (TRACERx deliberately selected for subclonal variants)

Figure 6: Comparison of Analytical Sensitivity for Single SNV Detection between TRACERx and Signatera



Analytical validation[6,14,29]

Signatera analytical validation was performed using two different sets of titration samples built from a) mononucleosomal DNA from cancer cell lines, and b) a commercially available mutation mixture from SeraCare, "SeraseqTM ctDNA Mutation Mix v2."

Mononucleosomal DNA from three cancer cell lines, including two breast cancer cell lines (HCC2218, HCC1395) and one lung cancer cell line (NCI–H1395), were titrated into their matched normal B lymphoblast-derived counterparts (HCC2218–BL, HCC1395–BL, and NCI–H1395–BL, respectively). Titrations of tumor into normal mononucleosomal DNA were made at average VAFs (based on DNA input) of 1%, 0.5%, 0.3%, 0.1%, 0.05%, 0.03%, 0.01%, 0.005%, 0%. Six primer pools were tested with replicate numbers from two to nine (for each pool)—increasing with the dilution factor. In addition, a commercially available control SNV mixture (SeraseqTM ctDNA Mutation Mix v2) was titrated from 0.5% to 0.005%. Starting allele fractions were confirmed by SeraCare by droplet digital PCR. Two primer pools were tested in triplicate on these mixtures.

The starting total input into library prep for each reaction was 15,000-20,000 haploid genome equivalents. SNV targets from the corresponding tumor DNA spike-in samples were amplified using the 16-plex-PCR assay primer pools. The mPCR products were tagged with sequencing barcodes, 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/target.

The sample-level performance was derived by calculating a binomial probability for detecting at least two clonal mutations at a given ctDNA level, assuming that the majority of the custom panels have between 10 to 16 clonal variants. As shown in Table 3, ctDNA would be detected in samples with ctDNA between tumor DNA concentration of 0.01% and 0.02% for >98% of samples. Reproducibility was calculated as the percent coefficient of variation (%CV) of the median VAF of positive targets. Sample-level performance calculated from orthogonal control samples from SeraCare is shown in Table 4.

Table 3: Sample-level performance calculated when at least 2 variants are detected from a set of 16 target SNVs

Tumor DNA Concentration (%)	Tumor DNA Concentration Range (%)	Sensitivity Per Sample (%)	CV of Median VAF (%)	Data Points (n)
0.00375	0.0025-0.005	44.7-70.8	69.1	501
0.0075	0.005-0.01	58.9-83.3	44.2	562
0.015	0.01-0.02	98.5-100.0	23.8	474
0.025	0.02-0.03	99.9-100.0	25.1	278
0.04	0.03-0.05	100	17.6	289
0.0625	0.05-0.075	100	7.8	153
0.0875	0.075-0.1	100	16.1	72
0.2	0.1-0.3	100	10.1	268
0.4	0.3-0.5	100	6.4	120
0.75	0.5-1.0	100	6.6	117

Table 4: Sample-level performance calculated from orthogonal control samples from SeraCare

Tumor DNA Concentration (%)	Sensitivity Per Sample(%)	CV of Median VAF (%)	Data Points (n)
0.005	35.0-59.9	33.8	90
0.01	81.3-96.1	51.0	90
0.03	100	25.5	90
0.05	100	16.2	90
0.1	100	14.3	90
0.2	100	14.6	90
0.5	100	9.4	90

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