natera™ | Signatera™
Residual disease test (MRD)

Seeing beyond the limit: Detect residual disease and assess treatment response

Summary of analytical and clinical data for Signatera™, the first ctDNA assay custom-built for MRD detection and 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, progress in blood-based monitoring for solid tumors has been limited by the rarity and heterogeneity of circulating tumor cells and the extremely low concentrations of circulating tumor DNA (ctDNA) in the blood.[3] With recent advances in next generation sequencing (NGS) technologies, there has been renewed interest in using ctDNA profiling as a tool for detecting residual disease, detecting relapse early, and assessing treatment response in solid tumors.[3,4]

Signatera™ is the first ctDNA assay that has been custom-built for detecting molecular residual disease (MRD) and assessing treatment response, with the ability to detect ctDNA at a variant allele frequency (VAF) of <0.1% of cell free DNA (cfDNA) from plasma.[5-9] This white-paper reviews the differences between Signatera and other ctDNA detection assays, summarizes data from the analytical and clinical validation of Signatera, and presents potential research applications of Signatera in clinical studies.

The Signatera approach

Currently available assays for detecting ctDNA from patient plasma tests for a fixed panel of hotspot or actionable mutations. Given the heterogeneity of cancer, even large generic panels targeting up to more than a hundred of genomic loci might detect only a few mutations from a given individual's primary tumor.[10-12] Mutations identified in these panels may not be tumor derived, making such approaches less specific.[10-12] Additionally, the plasma-level VAF limit of detection (LOD) starts at approximately 0.1–1% for these conventional technolgies, which is 10–fold lower than the 0.01% VAF LOD achieved with Signatera.[13-17]

For the Signatera approach, somatic 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 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. Compared to other ctDNA approaches, 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%. 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]

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 and indel variants specific to each patient are bioinformatically identified and prioritized. Next, 16 somatic single-nucleotide and indel variants for multiplex PCR primer design are selected based on several factors, including the clonality, detectability, and frequency of the variants identified in the tumor tissue DNA. For longitudinal surveillance, cell free DNA (cfDNA) libraries are prepared from each 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.

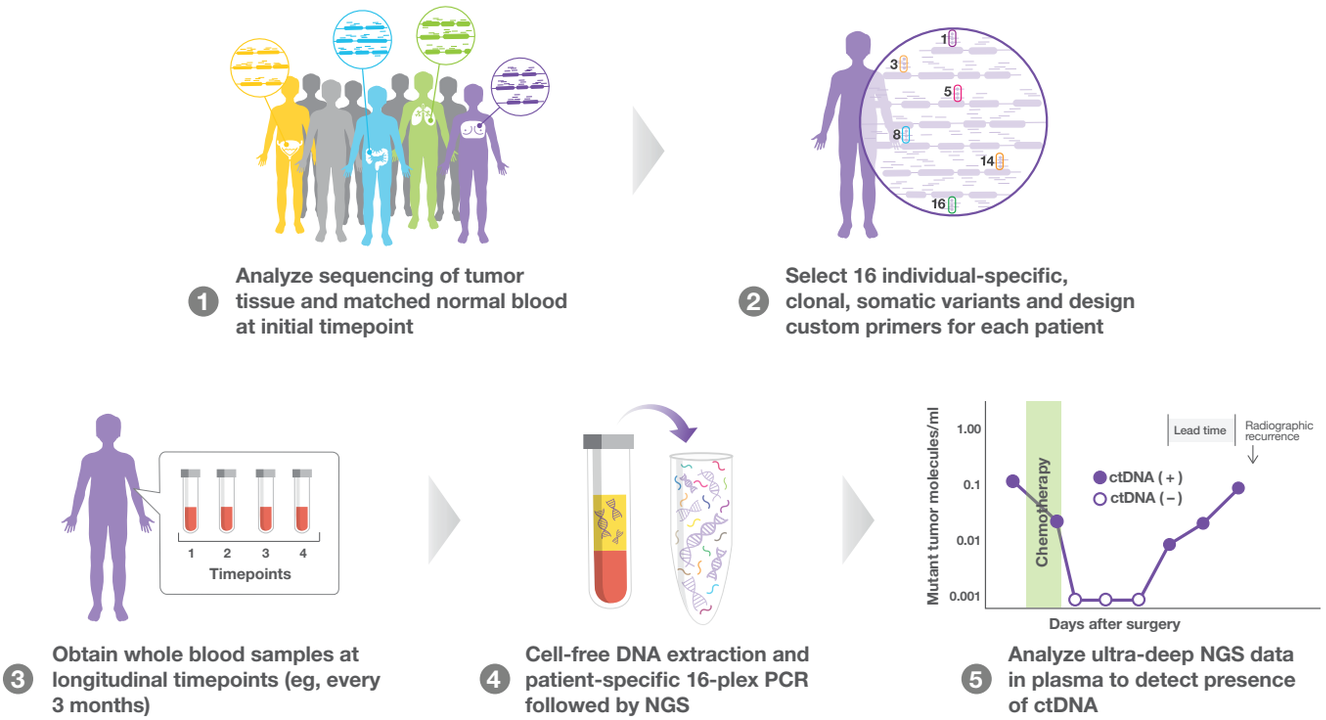
Step 2: The top 16 somatic variants are selected based on clonality, detectability, and frequency of mutations. Multiple-PCR compatible primers are designed for each of the 16 somatic variants.

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



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 1, 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 2.

Table 1: 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 2: 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

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 generated 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[19-21]
- 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

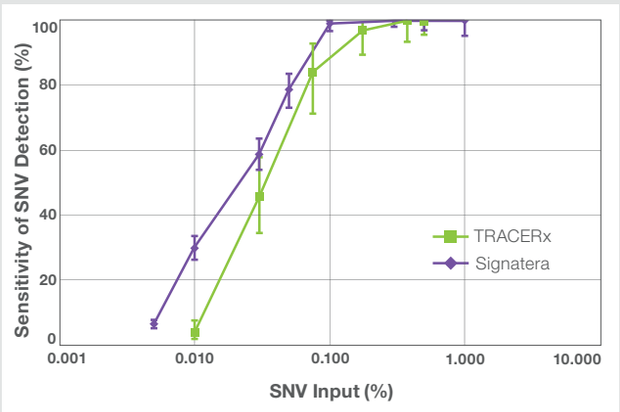
Due to the reasons above, Natera has developed other methods in the workflow to optimize the sensitivity and specificity of Signatera without use of molecular barcoding.

Signatera prototype in the TRACERx study

A prototype of the Signatera assay was used in the non-small cell lung cancer (NSCLC) TRACERx study to phylogenetically profile and detect cancer tumor DNA recurrence up to 346 days earlier than standard radiological confirmed relapse.[5] A sensitivity of 93%, with no false positives, was demonstrated for early relapse detection in patients with stages I–III lung cancer. 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 generic 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. 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 2, demonstrates the sensitivity improvements that have been made since the TRACERx study at tumor DNA concentration levels below 0.5%.[5,6,18]

Figure 2: Comparison of Analytical Sensitivity for Single SNV Detection between TRACERx and Signatera



Pan-cancer clinical summary of Signatera

Since the TRACERx study in lung cancer, the potential clinical usefulness of Signatera has also been demonstrated in colorectal, breast, and bladder cancers (tables 3-5, figures 3-5).[7-9]

Table 3: Study overview of Signatera in colorectal cancer[7]

	Colorectal Cancer Study Overview
N	125
CRC stage	I to III
Prior treatment received	Received curative surgery and adjuvant chemotherapy
# of blood time points	795
# of relapses	16
Average lead time	8.7 Months
Median lead time	10.1 Months
Maximum lead time	16.5 Months
Specificity	98%

Table 4: Study overview of Signatera in breast cancer[9]

	Breast Cancer Study Overview
N	49
Breast cancer stage	I to III
Subtype (n)	HR+/HER2-=34 HER2+= 8 TNBC= 7
Prior treatment received	Adjuvant chemotherapy within 3 years of study entry
# of blood time points	208
Average lead time	9.5 months
Median lead time	8.9 months
Maximum lead time	2 years
Specificity	100%

Table 5: Study overview of Signatera in muscle-invasive bladder cancer (MIBC)[8]

	MIBC Study Overview
N	68
Prior treatment received	Received neoadjuvant or first-line chemotherapy before cystectomy
# of blood time points	656
# of relapses	16
Average lead time	2.8 Months
Median lead time	3.2 Months
Maximum lead time	8.2 Months
Specificity	100%

Figure 3. Relapse-free survival of local or regionally advanced CRC patients, stratified by ctDNA status
Detection of positive ctDNA after surgery and after adjuvant chemotherapy is associated with higher risk of relapse.

- Molecular relapse was detected at an average of 8.7 months before clinical relapse

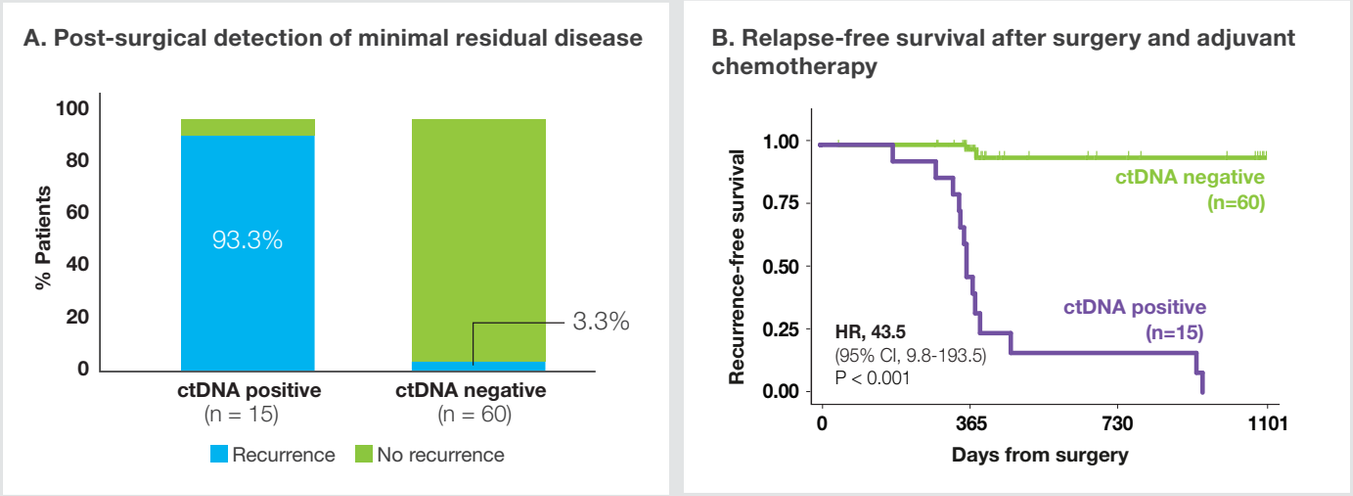


Figure 4. Relapse-free survival of local or regionally advanced breast cancer patients, stratified by ctDNA status
Detection of positive ctDNA after surgery and after adjuvant chemotherapy is associated with higher risk of relapse.

- Molecular relapse was detected at an average of 9.5 months

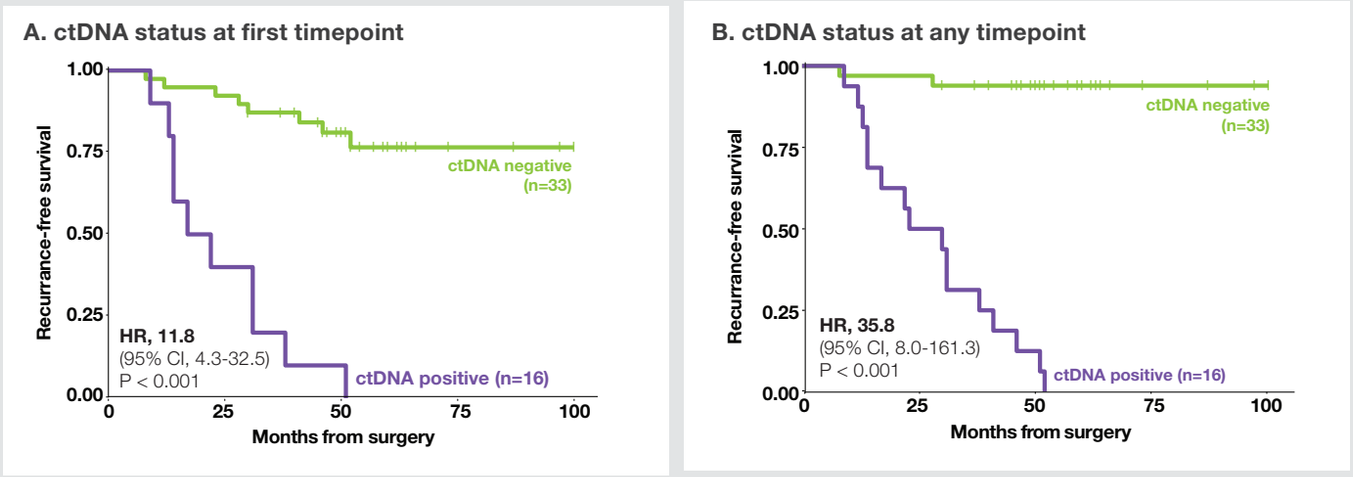
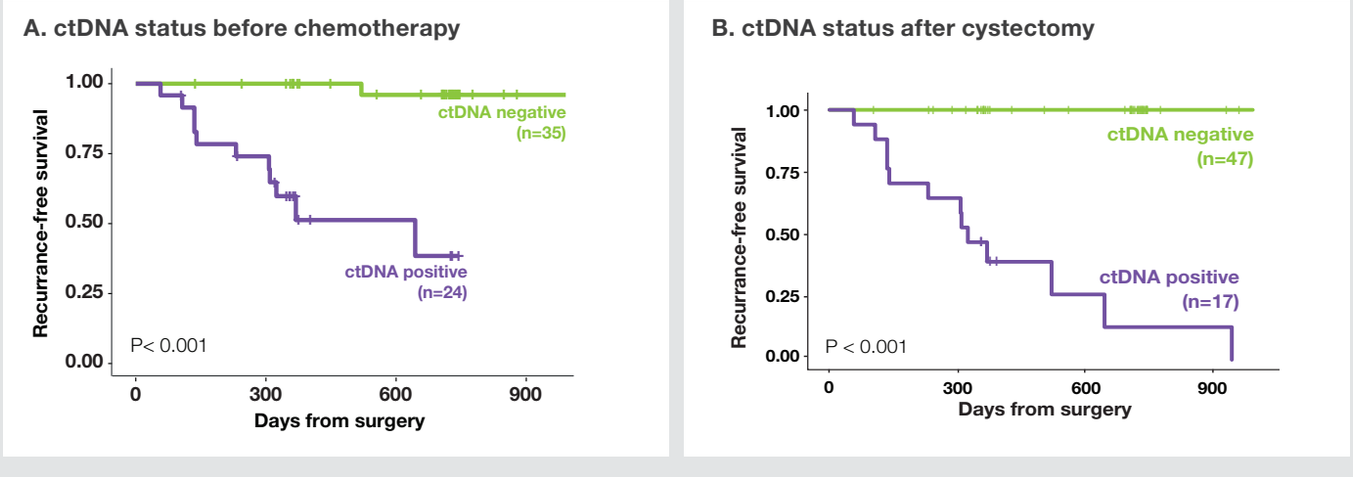


Figure 5. Relapse-free survival of MIBC patients, stratified by ctDNA status
Significantly shorter relapse-free rates were observed for patient who are ctDNA-positive at diangnosis and post cystectomy.

- Molecular relapse was detected at an average of 2.8 months before clinical relapse



Potential applications of Signatera in the clinical setting and in drug development

Unlike other ctDNA assays that are being developed for early cancer detection or as a tissue biopsy replacement for identifying actionable mutations in metastatic disease, applications of Signatera ctDNA analysis in the clinical setting could include predicting the likelihood of relapse at diagnosis, monitoring response to neoadjuvant treatment, detecting minimal residual disease, monitoring for recurrence after adjuvant treatment, or monitoring for treatment resistance (table 6, figure 6).

In drug development, ctDNA status determined by Signatera may potentially be used as an entry criterion for clinical trial enrollment. Enriching for patients based on ctDNA status, which correlates with the likelihood of relapse or treatment response, may have implications in decreasing sample sizes, shortening time to completion, minimizing total cost, and increasing the likelihood of trial success. Additionally, ctDNA detection at different time points during the trial may potentially complement Response Evaluation Criteria in Solid Tumors (RECIST) as a measurement of treatment response.

Conclusions

Advances in next-generation sequencing technology have now made it possible to detect low quantities of ctDNA.[4,13] Signatera is the first individualized, pan-cancer ctDNA platform that has potential applications in oncology for detecting MRD and monitoring treatment response with a low limit of ctDNA detection. Signatera has been analytically validated as a highly sensitive and specific platform for detecting and quantifying patient-specific, somatic SNVs and indels at a VAF of 0.01%.[6] Analytical and clinical results across different tumor types suggest that Signatera may have wide-ranging clinical and drug development applications.[5-9]

Table 6: Proposed applications of Signatera

Applications	Signatera results	Implications	Trial designs	Example
Molecular residual disease after surgery	ctDNA-positive	Increased rate of relapse	Trials combining novel therapy and adjuvant therapy	Addition to SOC of anti-PD-1/PD-L1 vs placebo for NSCLC patients who are ctDNA-positive
	ctDNA-negative	Decreased rate of relapse	Trials to avoid or de-escalate treatment	
Recurrence monitoring	ctDNA-positive	Increased rate of relapse	Trials to increase surveillance during recurrence monitoring, showing correlation to outcomes	Imaging every 3 months vs 6 months in ctDNA-positive patients
Molecular relapse after adjuvant treatment	ctDNA-positive without evidence of clinical relapse	>98% certainty for clinical relapse without additional therapy	Trials to treat with additional therapy prior to clinical relapse	Extending duration of adjuvant therapy in patients who are persistently ctDNA-positive after finishing standard course of treatment
Treatment monitoring	ctDNA levels increasing or unchanged	Increased rate of therapy failure	Trials to correlate ctDNA-negative status after treatment with decrease in RFS or improvement in PFS	ctDNA to predict response to I-O therapy, distinguish pseudo-progression from true progression, and determine early trial readout
	ctDNA levels decreasing or undetectable	Decreased rate of therapy failure		

Figure 6: Applications of Signatera in detecting ctDNA

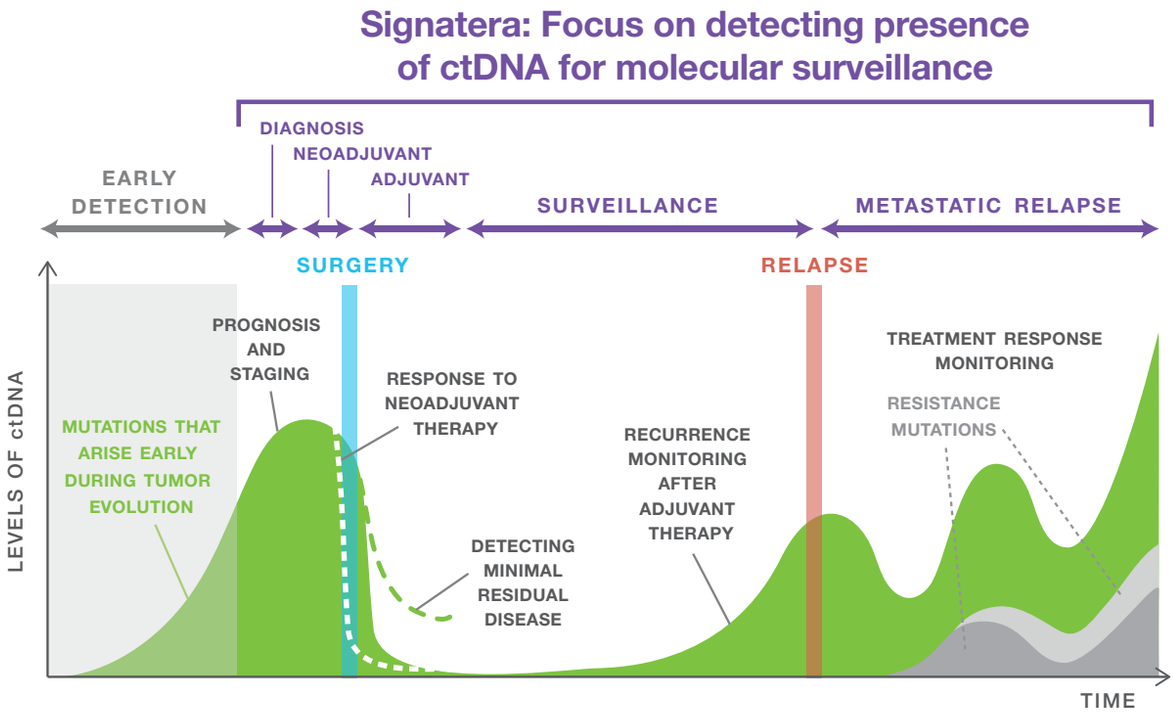
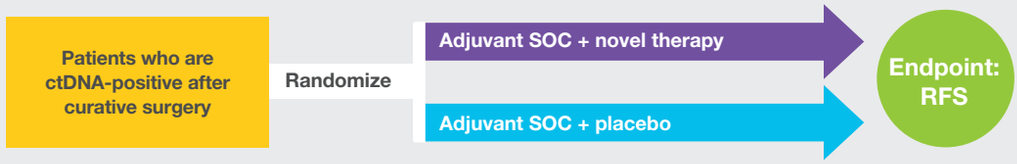
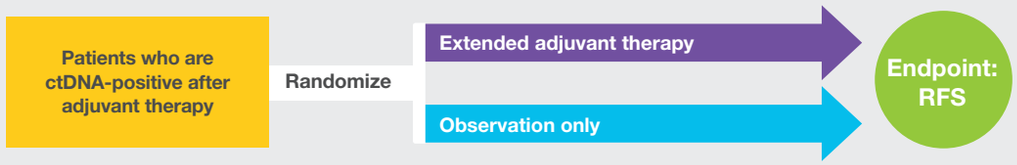


Figure 7: Potential clinical trial designs with Signatera

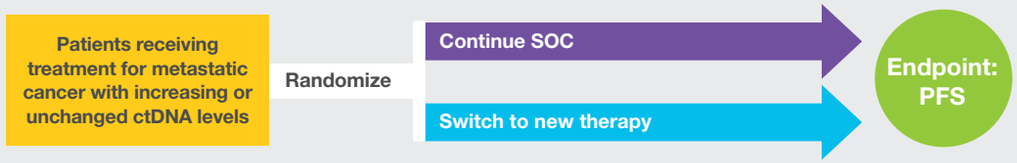
Trials to combine novel therapy with standard of care (SOC) adjuvant therapy for patients with high risk of clinical relapse



Trials to identify early-stage breast cancer patients with high risk of clinical relapse and treat with extended adjuvant therapy



Trials in metastatic cancer to correlate ctDNA-negative status after treatment with outcome



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