Molecular Diagnosis & Therapy (2023) 27:753–768
https://doi.org/10.1007/s40291-023-00670-1

ORIGINAL RESEARCH ARTICLE

# Personalized Cancer Monitoring Assay for the Detection of ctDNA in Patients with Solid Tumors

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Accepted: 27 July 2023/ Published online: 26 August 2023


## Abstract

**Background** Highly sensitive molecular assays have been developed to detect plasma-based circulating tumor DNA (ctDNA), and emerging evidence suggests their clinical utility for monitoring minimal residual disease and recurrent disease, providing prognostic information, and monitoring therapy responses in patients with solid tumors. The Invitae Personalized Cancer Monitoring™ assay uses a patient-specific, tumor-informed variant signature identified through whole exome sequencing to detect ctDNA in peripheral blood of patients with solid tumors.

**Methods** The assay's tumor whole exome sequencing and ctDNA detection components were analytically validated using 250 unique human specimens and nine commercial reference samples that generated 1349 whole exome sequencing and cell-free DNA (cfDNA)-derived libraries. A comparison of tumor and germline whole exome sequencing was used to identify patient-specific tumor variant signatures and generate patient-specific panels, followed by targeted next-generation sequencing of plasma-derived cfDNA using the patient-specific panels with anchored multiplex polymerase chain reaction chemistry leveraging unique molecular identifiers.

**Results** Whole exome sequencing resulted in overall sensitivity of 99.8% and specificity of > 99.9%. Patient-specific panels were successfully designed for all 63 samples (100%) with ≥ 20% tumor content and 24 (80%) of 30 samples with ≥ 10% tumor content. Limit of blank studies using 30 histologically normal, formalin-fixed paraffin-embedded specimens resulted in 100% expected panel design failure. The ctDNA detection component demonstrated specificity of > 99.9% and sensitivity of 96.3% for a combination of 10 ng of cfDNA input, 0.008% allele frequency, 50 variants on the patient-specific panels, and a baseline threshold. Limit of detection ranged from 0.008% allele frequency when utilizing 60 ng of cfDNA input with 18–50 variants in the patient-specific panels (> 99.9% sensitivity) with a baseline threshold, to 0.05% allele frequency when using 10 ng of cfDNA input with an 18-variant panel with a monitoring threshold (> 99.9% sensitivity).

**Conclusions** The Invitae Personalized Cancer Monitoring assay, featuring a flexible patient-specific panel design with 18–50 variants, demonstrated high sensitivity and specificity for detecting ctDNA at variant allele frequencies as low as 0.008%. This assay may support patient prognostic stratification, provide real-time data on therapy responses, and enable early detection of residual/recurrent disease.

## 1 Introduction

Early detection of residual or recurrent disease, reliable monitoring of treatment response, and accurate assessment of prognostic risk are essential to the clinical management of cancer. The ability to monitor clinical outcomes after surgical resection of tumors or therapy has been limited to imaging technology or to leveraging serum biomarkers [1, 2]. Currently, imaging technology has limited sensitivity

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**Key Points**

The Invitae Personalized Cancer Monitoring™ assay uses a patient-specific, tumor-informed variant signature to detect circulating tumor DNA leveraging unique molecular identifiers, anchored multiplex polymerase chain reaction (AMP™) technology, stringent quality control, and flexible design of patient-specific panels with 18–50 variants.

Analytical validation of the assay demonstrated greater than 99% sensitivity and specificity for both tumor whole exome sequencing and tumor-informed circulating tumor DNA detection, including sensitive detection of circulating tumor DNA at variant allele frequencies as low as 0.008%.

A research-use version of this assay has supported both a phase III clinical trial assessing therapy responses in patients with resectable lung cancer and the TRACERx study, which recently demonstrated the feasibility of using circulating tumor DNA detection for predicting prognoses and monitoring residual or recurrent disease in patients with early-stage lung cancer.

and specificity, especially for detecting small tumors, and serum biomarkers are not sufficiently specific and accurate for diagnosing cancer as many factors can influence their levels [3–6]. For optimal clinical management, highly sensitive molecular biomarkers are needed to detect minimal residual disease (MRD) [i.e., the small fraction or number of cancer cells remaining after a therapeutic intervention] and recurrent events as early as possible.

Cell-free DNA (cfDNA) is fragmented DNA shed by cells in the body into the bloodstream and other body fluids. The cfDNA released from tumors is specifically referred to as circulating tumor DNA (ctDNA) and comprises only a fraction of total cfDNA. Highly sensitive molecular assays have been developed to detect plasma-based ctDNA that, unlike repetitive serial surgical biopsies, require only one initial surgical biopsy followed by less-invasive serial ctDNA monitoring using peripheral blood. This approach overcomes several historical obstacles to monitoring, such as invasive biopsy procedures, insufficient surgical biopsy tissue available for repeat testing, sampling bias due to intra-tumor heterogeneity, and the inability to sample all metastatic tumors [7, 8]. However, ctDNA methods need to overcome limitations such as false-positive results due to low-level mosaic germline variants, clonal hematopoiesis of indeterminate potential (CHIP) variants (i.e., somatic mutations that accumulate in normal hematopoietic cells with age) [9], and other technical challenges, such as the failure to detect the tumor signature when ctDNA allele frequencies (AFs) are very low.

Next-generation sequencing (NGS) technology has allowed for high-throughput parallel sequencing of cfDNA, enabling ultra-high coverage per sequencing target and detection of multiple molecular markers (i.e., somatic variants) by a single preparation with a single assay. Next-generation sequencing-based ctDNA assays, particularly those incorporating unique molecular indexes (UMIs), have higher accuracy and sensitivity and lower limits of detection (LODs) than other molecular assays [10, 11]. The use of UMIs in NGS library preparation, allowing correction of errors introduced during polymerase chain reaction (PCR) and sequencing, has been shown to increase the accuracy and sensitivity of NGS, especially for low allele frequency (AF) variant detection [12, 13]. Next-generation sequencing that incorporates anchored multiplex PCR (AMP™) chemistry enables strand-specific assessment of loci and accurate detection and quantification of high confidence variant calls [14]. Compared with traditional PCR techniques, AMP chemistry reduces PCR amplification bias and allows for more complete and even coverage for genes associated with pseudogenes [14].

Additional studies have shown that ctDNA detection is more sensitive and specific when tracking multiple variants than when tracking a single driver mutation [1] and that ctDNA assays that use a panel of patient-specific tumor-informed biomarkers have lower LODs and higher assay specificity than those that use fixed somatic variant panels [15]. Variant signatures developed from truncal mutations of a primary tumor are more likely to be detected in plasma than subclonal mutations and less likely to be influenced by clonal evolutionary pressures, ensuring a higher likelihood that the variant will be present for the lifetime of the tumor. This allows ctDNA to be detected from both the primary tumor and any metastatic lesions without the need for additional tumor biopsies [16].

A pan-cancer, tumor-informed, long-term monitoring assay that incorporates NGS libraries featuring both UMIs and AMP chemistry, Invitae Personalized Cancer Monitoring, has been developed to provide personalized ctDNA detection from the peripheral blood of patients with solid tumors. A research-use version of this assay has been utilized to support the CheckMate 816 phase III clinical trial in assessing neoadjuvant nivolumab plus chemotherapy in resectable non-small cell lung cancer [17]. It has also supported the TRACERx [TRAcking Cancer Evolution through treatment (Rx)] study in predicting prognoses and monitoring residual or recurrent disease, as well as in extracting clonality from ctDNA results, in patients with early-stage non-small cell lung cancer [18]. Although the TRACERx publication reported a partial analytical validation of the research-use assay with panels containing 50 patient-specific

somatic variants, here we describe the complete analytical validation of the Invitae Personalized Cancer Monitoring assay for detecting ctDNA in peripheral blood using panels containing 18–50 variants.

## 2 Subjects and Methods

### 2.1 Study Design

Invitae Personalized Cancer Monitoring assay (Fig. 1) has two components: whole exome sequencing (WES) and ctDNA detection. Matched tumor and germline specimens from a patient undergo WES to identify tumor-specific somatic variants, which are then used to design a patient-specific panel (PSP) that captures the patient's unique tumor variant signature. Because the PSP design uses both a tumor specimen and a germline specimen from the same individual, germline, CHIP, and other artifact variants present in the germline specimen are subtracted, allowing personalized monitoring of somatic variants present only in the patient's tumor. Next-generation sequencing libraries featuring both UMIs and AMP chemistry are generated from cfDNA using the PSPs, and ctDNA is then analyzed by a proprietary ctDNA-calling algorithm with built-in error correction and sequencing noise modeling. For continued observation of ctDNA levels, sequencing data from patient plasma specimens, serially collected at monitoring intervals recommended by guidelines for a specific tumor type, can be analyzed.

This assay was validated in a laboratory with both College of American Pathologists and Clinical Laboratory Improvement Amendments accreditation (Invitae MetroPark, Iselin, NJ, USA). To validate both WES and the ctDNA detection component of the assay, separate WES and cfDNA-derived libraries were created from combinations of formalin-fixed paraffin-embedded (FFPE) tumor specimens, germline specimens (i.e., peripheral blood, buffy coat, and normal tumor-adjacent FFPE specimens), commercially purchased reference samples, and cfDNA specimens. The WES libraries were then used to assess WES accuracy, limit of detection (LOD), limit of blank (LOB) for PSP design, and reproducibility and precision. The cfDNA-derived libraries were used to measure the LOD, accuracy, and reproducibility and precision of ctDNA detection. End-to-end testing was also performed to validate the entire assay incorporating various DNA inputs, preparation times, operators, and sequencers. All studies complied with applicable federal, state, and local regulatory requirements.

### 2.2 Specimens and Commercial Samples

A total of 259 specimens and samples, 250 unique human specimens and 9 commercially purchased reference samples, were used to validate the Invitae Personalized Cancer Monitoring assay (Fig. 1 of the Electronic Supplementary Material [ESM]). The unique human specimens comprised 77 FFPE tumor specimens from various tumor diagnoses including breast cancer, colorectal cancer, lung cancer, and melanoma covering primary, relapsed, and metastatic neoplasms; 39 germline FFPE histologically normal tumor-adjacent specimens; 16 germline peripheral blood specimens; 19 bone marrow specimens from patients with diagnosed hematological malignancies; 4 germline buffy coat specimens; and 95 peripheral blood specimens for ctDNA detection. For 69 of the specimens (50 FFPE tumor specimens and all 19 bone marrow specimens), externally extracted DNA was obtained from an inter-laboratory exchange and had been

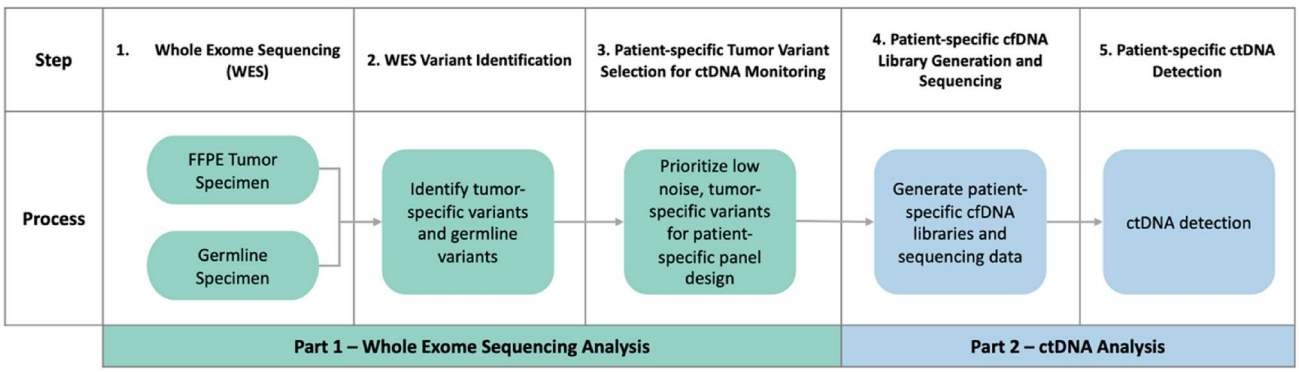



**Fig. 1** Workflow for the Invitae Personalized Cancer Monitoring assay. This tumor-informed assay utilizes whole exome sequencing of matched tumor and germline specimens to identify tumor-specific variants. A proprietary algorithm selects tumor-specific variants to design a patient-specific panel. The designed panel is then used to generate next-generation sequencing libraries, featuring anchored multiplex polymerase chain reaction chemistry, and unique molecular identifiers, from cell-free DNA (cfDNA) extracted from the patient's plasma specimen. From the sequencing data, stringent data quality-control measures and a proprietary circulating tumor DNA (ctDNA)-calling algorithm are used to assess the ctDNA status in the patient's specimen. *FFPE* formalin-fixed paraffin-embedded

previously characterized: 40 had been characterized by two outside academic institutions using a targeted 147-gene panel for solid tumors and a targeted 68-gene panel for hematologic malignancies; 29 had been characterized internally using a targeted 67-gene solid tumor panel (Archer VariantPlex Solid Tumor; Integrated DNA Technologies, Coralville, IA, USA). Human specimens were either purchased through commercial vendors or acquired through an inter-laboratory exchange. All human specimens and corresponding clinical data were de-identified per the Health Insurance Portability and Accountability Act privacy rule prior to acquisition.

Commercially purchased reference samples comprised six well-characterized samples from the Genome in a Bottle project at the National Institute of Standards and Technology (i.e., NA24149, NA24631, NA24694, NA24695, NA24385, NA12878) and three synthetic tumor samples: SeraSeq Tumor Mutation DNA Mix AF10, Seraseq Tri-Level Tumor Mutation DNA Mix, and SeraSeq Myeloid Mutation DNA Mix (SeraCare, Milford, MA, USA). Together these specimens and samples resulted in 1349 libraries (603 WES libraries and 746 cfDNA-derived libraries) that were used across the validation studies.

### 2.3 Specimen Collection

FFPE tumor specimens and histologically normal FFPE specimens were processed in pathology laboratories, and peripheral blood specimens were drawn by a routine phlebotomy. Tumor specimens were required to have an estimated tumor percentage of ≥ 10% by a pathology review. Peripheral blood was collected in EDTA tubes, and buffy coats were isolated via centrifugation for extraction of genomic DNA for germline WES. Peripheral blood was also collected in Cell-Free DNA BCT Streck tubes (Streck, La Vista, NE, USA) for isolation of cfDNA for ctDNA detection. For ctDNA detection, ≥ 4 mL of peripheral blood, typically yielding ≥ 2 mL of plasma, was needed for each test.

### 2.4 DNA Extraction, Library Preparation, and NGS

The KingFisher Flex extraction system (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract DNA from FFPE and peripheral blood specimens utilizing the Maxwell HT DNA FFPE Isolation System (Promega, Madison, WI, USA) and Maxwell HT 96 gDNA Blood Isolation System (Promega), respectively, according to the manufacturer's protocol. Extracted tumor DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA); DNA with a concentration > 1 ng/μL and a KAPA Quant 129/130 bp to PicoGreen Quant-iT ratio > 0.1 was considered acceptable. Extracted germline DNA was quantified using the KAPA Human Genomic DNA Quantification and QC Kit (Roche, Basel, Switzerland); DNA with a concentration > 3 ng/μL was considered acceptable. The DNA input amount used for WES was 50–200 ng for both tumor and germline specimens.

Peripheral blood collected in Streck tubes (Streck, La Vista, NE, USA) for cfDNA isolation was processed within 7 days of collection and stored at ambient temperature (15–25 °C) until plasma separation. Plasma was separated using a two-step centrifugation protocol with the following conditions: 300×*g* for 20 minutes at 25 °C followed by a second centrifugation at 5000×*g* for 10 min at 25 °C. Cell-free DNA was then isolated from the plasma using the Maxwell RSC Circulating Cell-Free DNA Purification Kit (Promega) according to the manufacturer's instructions. Cell-free DNA concentrations ≥ 0.10 ng/μL by a fluorescence-based methodology were considered acceptable.

DNA underwent NGS library preparation featuring either hybridization capture for WES (Integrated DNA Technologies) or AMP chemistry with PSPs for ctDNA detection. Next-generation sequencing libraries were sequenced using Illumina NextSeq or NovaSeq sequencers with 2×150bp chemistry (Illumina, San Diego, CA, USA).

### 2.5 Generation of PSPs

Patient-specific panels were designed using the PCM, The Gentleman Pipeline (1.7.3-1+; Invitae, San Francisco, CA, USA). Tumor-specific somatic variant selection was performed using WES variant calls from FFPE tumor specimens and matched germline specimens. Variants identified in the matched germline specimens, including heterozygous and homozygous germline variants as well as low AF variants representing CHIP or mosaic germline variants, were subtracted from the variants identified in the tumor. Each PSP was generated using proprietary algorithms (Invitae) along with stringent quality-control (QC) measures to identify and rank tumor-specific variants that considered the somatic origin of the variants with a minimum AF of 5%, the predicted error rate for each variant, the quality and distance of primers to the variants, and whether the region surrounding the variants contained sequences that could cause a high error rate. Outlier AF variants that were likely artifacts were excluded. Primers for custom-designed PSPs were then designed to track up to 50 somatic variants for the detection of ctDNA.

### 2.6 Bioinformatic Data Processing and ctDNA Calling

Matched tumor-germline WES data were processed using the Integrated Genomic Toolkit, Capture Pipeline (v2.17.3+; Invitae), Briefly, reads were aligned to the GRCh37/hg19 reference genome using BWA-MEM [19]. Variant calling for single nucleotide variants (SNVs) and small insertions and deletions (indels) was performed by a proprietary variant caller. Data QC of WES included sequencing read depth QC (mean coverage depth of ≥ 150× for tumor WES and 90% exome coverage at 20× or greater for germline WES) and concordance between reported and determined sex.

The ctDNA sequencing data were processed using the PCM MRD Pipeline (v1.6.0-1 or later; Invitae). The ctDNA calling was performed by a previously described algorithm [18]. Data QC of ctDNA detection included identity QC (i.e., assessing single nucleotide polymorphism [SNP] signatures in FFPE tumor, germline, and plasma specimens to ensure that all three specimens were from the same individual), sequencing reads QC (i.e., counting deep reads post-error correction using UMIs with five or more regular sequencing reads; the number of deep reads covering somatic target variants had to meet ≥ 10,000 for the baseline threshold and ≥ 30,000 for the monitoring threshold for 50 variant PSPs, and these cut-offs were adjusted accordingly for PSPs with < 50 variants), and variant QC (i.e., using noise modeling and outlier AF filtering). The total number of deep reads of observed variants that passed the filters were tested against expected deep reads of all targeted variants using a one-sided Poisson test; $p$ value < 0.01 signified ctDNA detection for the baseline threshold, and $p$ value < 0.001 signified ctDNA detection for the monitoring threshold. Confidence intervals (CIs) were calculated using Epitools with the Jeffreys method (https://epitools.ausvet.com.au/).

## 3 Results

### 3.1 Validation of Tumor WES

Whole exome sequencing was analytically validated using 603 libraries generated from 118 specimens and samples: 113 unique human specimens (56 tumor FFPE specimens, 38 germline FFPE histologically normal specimens, and 19 bone marrow specimens), two Genome in a Bottle samples (NA24385 and NA12878), and three synthetic SeraSeq samples (Tumor Mutation DNA AF Mix, Tri-Level Tumor Mutation DNA Mix, and Myeloid Mutation DNA Mix). Sequencing results showed that 99.3% (95% CI 98.6–99.9) of the tumor samples in 588 tumor libraries achieved ≥ 150× mean coverage across target regions in the exome and that 15/15 (100%) of germline specimens met data QC. Reported sex was consistent with the exome-determined sex in 100% of sequencing libraries. One specimen had undetermined sex by the algorithm, but male sex was verified with a low Y chromosome dosage.

#### 3.1.1 Accuracy of WES

The 69 previously characterized tumor specimens (50 FFPE tumor specimens and 19 bone marrow specimens) were sequenced to assess the sensitivity of WES. Four of the specimens had known negative results, and the remaining 65 had been reported to include 7609 variants. Whole exome sequencing of these specimens showed sensitivity of 99.8% (95% CI 99.7–99.9) with 7594 of the 7609 variants detected. The remaining 15 variants were not detected (i.e., were outside of the reportable range of WES) either because of a low-coverage region in the exome or because variants with low AF (< 20%) were not called by the bioinformatics pipeline (Table 1 of the ESM).

To further assess the sensitivity and specificity of WES, the well-characterized NA12878 sample was sequenced, and detected variants were compared to the benchmark high-confidence variant calls made by Genome in a Bottle. Results showed sensitivity of > 99.9% (95% CI 99.9–100) and specificity of > 99.9% (95% CI 98.9–100).

#### 3.1.2 Limit of Detection of WES: Tumor Content for PSP Design

An LOD study to determine the lower limit of tumor content that could be used for successful PSP design was conducted using 14 FFPE specimens: seven FFPE tumor specimens with tumor content ≥ 20% and seven FFPE tumor-adjacent matched germline specimens. To simulate results from tumors with < 20% tumor content, two dilutions of tumor DNA were generated using matched germline genomic DNA from the same individual to create 14 contrived samples with tumor content of ~15% and ~7.5%. DNA from the seven original tumor specimens and the 14 contrived samples were processed in triplicate, and DNA from the seven original germline specimens was processed in duplicate, at input amounts of 50 ng, 100 ng, and 200 ng to create 231 WES sequencing libraries (Table 1).

Of the 231 libraries, 230 (99.6%) passed all data QC. The single sample that did not meet QC had a mean coverage just below the cut-off and was conditionally passed

**Table 1** Design for the study on the limit of tumor content for panel design

| Tumor content | Genomic DNA input amount (no. of patients × no. of library replicates) | | |
|---|---|---|---|
| | 50 ng | 100 ng | 200 ng |
| ≥20% | 7 patients × 3 | 7 patients × 3 | 7 patients × 3 |
| ~15% (dilution 1) | 7 patients × 3 | 7 patients × 3 | 7 patients × 3 |
| ~7.5% (dilution 2) | 7 patients × 3 | 7 patients × 3 | 7 patients × 3 |
| Tumor-adjacent tissue only | 7 patients × 2 | 7 patients × 2 | 7 patients × 2 |
| Subtotals | 77 libraries | 77 libraries | 77 libraries |

Seven original tumor specimens with tumor content ≥20% were each diluted with matched germline genomic DNA to create seven contrived samples with ~15% tumor content and seven with ~7.5% tumor content. DNA from all 21 tumor specimens/samples were processed in triplicate and from seven matched normal tumor-adjacent specimens were processed in duplicate at three genomic DNA input amounts: 50 ng, 100 ng, and 200 ng

and included in the analysis; therefore, all 231 libraries were eligible for panel design. Sequencing revealed that two of the seven tumor specimens had a low somatic variant burden and failed to design a panel. Thus, PSP success rates were evaluated in the 135 libraries from the remaining five tumor specimens and ten corresponding contrived DNA samples. Patient-specific panels were successfully designed from 100% of the libraries with tumor content ≥ 20% (63/63), 80% of the libraries with tumor content between 10 and 20% (24/30), and 54% of the libraries with tumor content < 10% (23/42). DNA input amounts did not appear to impact the PSP design, as success rates were comparable whether 50 ng, 100 ng, or 200 ng were used (Fig. 2).

#### 3.1.3 Limit of Detection of WES: Variant AF

A second LOD study was conducted to define the lower limit of AF for variant detection. Two groups of samples were sequenced: (1) two synthetic SeraSeq reference samples (Seraseq Tri-Level Tumor Mutation DNA Mix and SeraSeq Myeloid Mutation DNA Mix) with known variant AFs of 4%, 7%, 5%, 10%, and 15% across 61 known SNVs and indels and (2) seven contrived samples of NA12878 and NA24385 mixed at ratios of 10/90, 20/80, 30/70, 50/50, 70/30, 80/20, and 90/10 to create expected low AFs of 5%, 10%, and 15%. The two synthetic SeraSeq samples were processed in triplicate and the contrived samples processed in singlicate.

Sequencing of the synthetic SeraSeq samples revealed sensitivity of > 99.9% (95% CI 97.6–100) for the detection of variants with an AF ≥ 10%, and reduced sensitivity of 93.3% (95% CI 86.1–97.4) for the detection of variants with AF of 5–7% (Table 2 of the ESM). Sequencing results for the admixtures were similar.

The contrived admixtures were further used to test the efficiency of variant calling by the bioinformatics pipeline at or near the 5% AF calling threshold. The data exhibited a normal distribution; 50% of variants with an expected AF of 5% were shown by sequencing to have an AF ≥ 5%, and 100% of variants at an expectant AF ≥ 10% were called by the pipeline, irrespective of variant types. Therefore, the results confirmed the ability of the pipeline to detect variants at a 5% AF in real-world sample datasets.

#### 3.1.4 LOB of WES for Panel Design

An LOB study was conducted to determine if any false-positive variants (i.e., variants incorrectly identified by

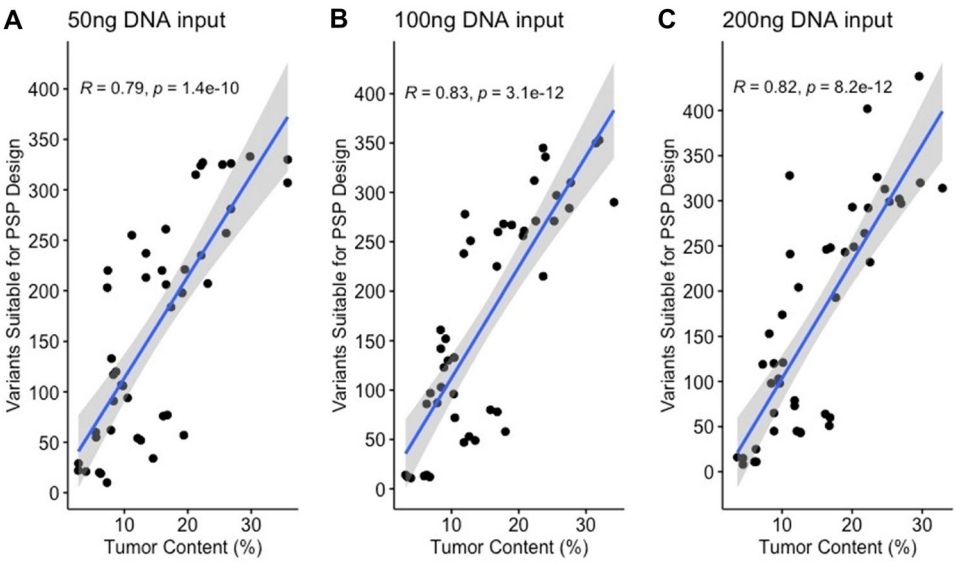



**Fig. 2** Effect of tumor content on variants suitable for patient-specific panel (PSP) design. Samples diluted to mimic low tumor cellular content that range from 5 to 35% were sequenced with whole exome sequencing at DNA input levels of (**A**) 50 ng, (**B**) 100 ng, and (**C**) 200 ng. The sequencing output was analyzed by the proprietary panel design algorithm to determine the expected range of variants suitable for the PSP design at the different tumor contents and DNA input levels. Results showed that the number of variants suitable for the PSP design was correlated with tumor content but not with the DNA input level

WES due to noise) would be chosen for inclusion on PSPs. Limit of blank was assessed using 30 FFPE histologically normal tumor-adjacent specimens. Two histologically normal replicates from each specimen were paired, with one replicate designated "tumor" and the other "germline." Each tumor and germline pair was processed in four replicates and sequenced on two NovaSeq sequencers to measure errors introduced during library preparation and sequencing, generating a total of 240 libraries from 120 tumor-germline paired specimens. All libraries met QC, with the exception that one specimen exhibited undetermined sex. A manual review revealed the absence of chromosome Y reads and the absence of X-chromosome heterozygosity, which was concordant with the previously reported female sex of the patient (likely a patient with Turner syndrome).

When the panel design was attempted with the sequencing results of 120 tumor-germline paired libraries, the pipeline identified only three variants across all libraries for potential inclusion in the PSPs (3/6000 possible variants, given 50 variants per panel and 120 design attempts), for a false detection rate of 0.05% (95% CI 0.01–0.13). Patient-specific panels failed for 100% (95% CI 97.9–100) of the 120 pairs used for LOB studies (Table 3A, B of the ESM).

#### 3.1.5 Reproducibility and Precision of WES

Reproducibility and precision were evaluated using three reference samples: Seraseq Tumor Mutation DNA Mix AF10, Seraseq Myeloid Mutation DNA mix, and NA12878. To evaluate reproducibility, NGS libraries of these samples were prepared in triplicate by two operators in three batches. To evaluate precision, NGS libraries were prepared in triplicate by a single operator and sequenced in a single sequencing run. All libraries passed QC.

Results showed reproducibility of 98.91% (181/183 variants; 95% CI 96.5–99.8) and precision of 99.45% (182/183 variants; 95% CI 97.5–99.9). The three variants not identified in the reproducibility and precision studies were all present in the raw data at levels slightly below the bioinformatics calling threshold of 5% AF. Higher concordance levels were observed for higher AF variants. Although concordance levels gradually decreased with lower AF ranges, > 95% overall concordance was observed with variants within 5–10% AF. Among the variant types, SNVs showed the highest overall concordance (> 99%), followed by duplications (~99%), deletions (~96%), and insertions (~93%) (Fig. 3).

### 3.2 Validation of ctDNA Detection

The ctDNA detection component of the assay was validated using 95 plasma specimens (including eight from known healthy individuals) and five reference samples (NA24385, NA24149, NA24631, NA24694, and NA24695). For the accuracy and end-to-end studies, an additional 21 FFPE tumor specimens and 21 matched germline specimens (four buffy coats, one germline FFPE histologically normal specimen, and 16 peripheral blood specimens) were processed to generate PSPs to assess the plasma specimens and additional contrived samples. A total of 746 libraries were generated from all specimens and samples.

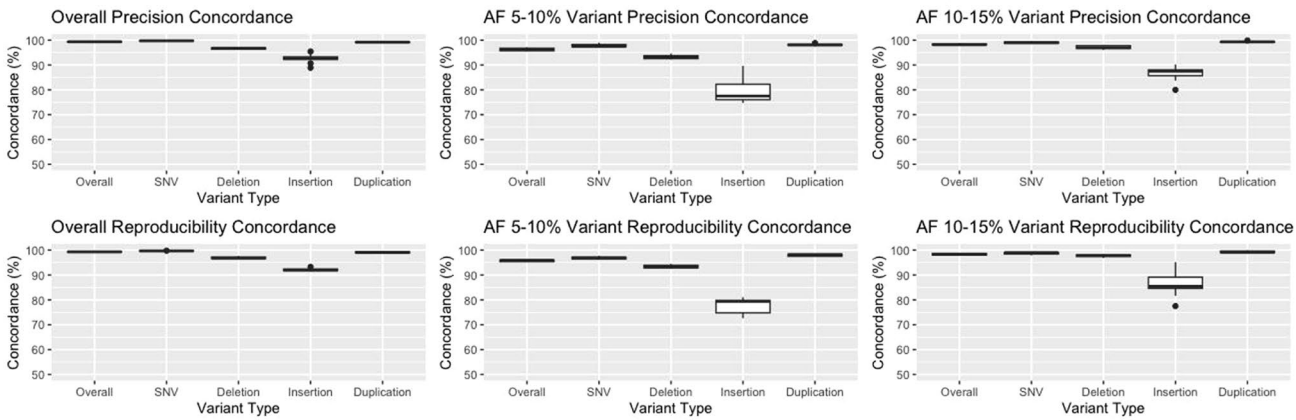



**Fig. 3** Precision and reproducibility of variants in whole exome sequencing. Reproducibility and precision were evaluated using three reference samples: Seraseq Tumor Mutation DNA Mix AF10, Seraseq Myeloid Mutation DNA mix, and NA12878. To evaluate reproducibility, next-generation sequencing libraries of these samples were prepared in triplicate by two operators in three batches. Overall precision and reproducibility concordance by overall variant types and four different variant types (single nucleotide variation [SNV], deletions, insertions, and duplications) are shown in the left panels, breakdowns to variant allele frequency (AF) to 5–10% is shown in the middle panels and 10–15% is shown in the right panels

### 3.2.1 Limit of Detection of ctDNA

An LOD study was conducted to define the limits of the assay to detect the ctDNA signal. Because of a limited volume of plasma available from real patients, LOD was evaluated by spiking sheared DNA from the NA24385 sample into background samples (i.e., NA24149, NA24631, NA24694, and NA24695) by mass-by-mass titrations, resulting in 48 combinations with different variant AFs that ranged from 0.003 to 0.1% using eight DNA input amounts (i.e., 2 ng, 4 ng, 5 ng, 10 ng, 20 ng, 40 ng, 60 ng, and 80 ng; Table 2). Each of the 48 contrived samples was assayed in duplicate for each of six predesigned 50-variant PSPs to create 576 NGS libraries, across different operators, preparation times, and sequencers. Two additional plasma specimens from healthy donors as well as pooled negative cfDNA samples served as negative controls.

Of the 576 NGS libraries, 562 (97.6%; 95% CI 96.1–98.6) passed all QC. The remaining 14 failed because of analytical metrics including low per-sample read counts and low-depth coverage. Using the 562 libraries that passed QC, a probit analysis was conducted to determine the LOD for the variant AF detected in at least 95% of the replicates (i.e., LOD95). The assay achieved sensitivity of > 99.9% (95% CI 94.9–100) at a variant AF of 0.03% and 10 ng of cfDNA input and sensitivity of > 99.9% (95% CI 93.3–100) at a variant AF of 0.008% and 60 ng of cfDNA input, when using a 50-variant NGS panel with a baseline threshold (Table 3).

Limits of detections were also assessed on PSPs with <50 variants under the same conditions used for the 50-variant panels (as fewer variants would potentially allow successful panel designs for samples with a lower tumor mutation burden, even if 50 variants were not detected). For this assessment, 398 NGS libraries from the original LOD design were computationally randomly downsized so that only 10, 14, 15, 16, 17, 18, 20, 30, and 40 tumor-specific variants were selected for panel design. To assess LODs at these lower PSP sizes, 3582 new data points were generated. Additionally, 46 known negative specimens were sequenced with the downsized panels to assess specificity when using fewer targeted variants. When thresholds for sequencing reads QC were adjusted proportionately to panel size, all libraries passed QC across panels targeting 16–50 variants for libraries generated using 10–60 ng of cfDNA input.

With PSPs of 18–50 variants and cfDNA input of 60 ng, the assay showed an LOD of 0.008% AF with > 99.9% (95%

**Table 2** Design of the study on the limit of ctDNA detection

| | cfDNA input amount | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 ng | 4 ng | 5 ng | 10 ng | 20 ng | 40 ng | 60 ng | 80 ng |
| AF (%) | 0.008 | 0.008 | 0.008 | 0.008 | 0.005 | 0.003 | 0.003 | 0.003 |
| AF (%) | 0.01 | 0.01 | 0.01 | 0.01 | 0.006 | 0.005 | 0.005 | 0.005 |
| AF (%) | 0.03 | 0.03 | 0.03 | 0.03 | 0.008 | 0.006 | 0.006 | 0.006 |
| AF (%) | 0.05 | 0.05 | 0.05 | 0.05 | 0.01 | 0.008 | 0.008 | 0.008 |
| AF (%) | 0.07 | 0.07 | 0.07 | 0.07 | 0.03 | 0.01 | 0.01 | 0.01 |
| AF (%) | 0.1 | 0.1 | 0.1 | 0.1 | 0.05 | 0.05 | 0.05 | 0.05 |

*AFs* allele frequencies, *cfDNA* cell-free DNA, *ctDNA* circulating tumor DNA

Forty-eight combinations of cfDNA input amounts and AFs were used to generate next-generation sequencing libraries in duplicate for each of six predesigned patient-specific panels, for a total of 576 libraries

**Table 3** Limit of detection at baseline and monitoring thresholds, by cell-free DNA input amount and the number of variants on the panel

| | Analytical threshold-input amount | Number of variants | |
|---|---|---|---|
| | | 18 Variants (%) | 50 Variants (%) |
| Variant allele frequency | Baseline, 10 ng | 0.05 | 0.03 |
| | Baseline, 20 ng | 0.05 | 0.03 |
| | Baseline, 30 ng | 0.05 | 0.008 |
| | Baseline, 60 ng | 0.008 | 0.008 |
| | Monitoring, 10 ng | 0.05 | 0.03 |
| | Monitoring, 20 ng | 0.05 | 0.03 |
| | Monitoring, 30 ng | 0.05 | 0.05 |
| | Monitoring, 60 ng | 0.05 | 0.01 |

CI 99.1–100) sensitivity using the baseline threshold. With the 18-variant panel and 10 ng of cfDNA input, the assay showed > 99.9% (95% CI 96.5–100) sensitivity at 0.05% AF (Table 3 and Fig. 4). When the monitoring threshold was used, panels with 18–50 variants and 60 ng of cfDNA input produced an LOD of 0.01% AF with > 99.9% (95% CI 99.1–100) sensitivity (Table 3 and Fig. 4). Specificity of > 99.9% (95% CI 94.7–100) was achieved for panels targeting ≥ 18 variants based on results from the 46 known ctDNA-negative plasma specimens. Panels with sizes below 18 variants resulted in an increased false-negative rate.

#### 3.2.2 Accuracy of ctDNA Detection

A two-part accuracy study was conducted to assess the ability of the assay to detect somatic variant signatures in samples with low ctDNA fractions. The first part of the accuracy study included four clinical plasma specimens with known variant AFs and eight plasma specimens from health donors. Because of the limited volume of plasma in the clinical specimens, cfDNA from each of the specimens was spiked into pooled cfDNA from the eight healthy donors through mass-by-mass titration to generate 36 contrived cfDNA samples with various variant AFs (i.e., 0.005%, 0.008%, 0.01%, 0.05%, and 0.1%). Next-generation sequencing libraries were generated in triplicate from the 36 contrived samples using 50-variant PSPs and three DNA input levels (10, 25, and 60 ng), for a total of 108 libraries. Of these libraries, 107 (99.07%, 95% CI 95.8–99.9) passed QC. The singular QC failure was because of the library not achieving sufficient sequencing depth.

Observed and expected ctDNA results are shown in Table 4. Results were concordant for all eight plasma samples from healthy donors (i.e., negative controls). However, ctDNA was not detected in four libraries in which detection had been expected; this was due to low input amounts at 10 ng and variant AFs of 0.008% and 0.01%, which were below the assay's LOD of 10 ng and 0.03% AF, as defined in the LOD study. Results of this portion of the accuracy study showed specificity of > 99.9% (8/8, 95% CI 73.8–99.9) and overall sensitivity of 95.7% (88/92, 95% CI 90.0–98.5) for ≥ 10 ng of cfDNA input and ≥ 0.008% AF (Table 4 and Table 4 of the ESM).

The second part of the accuracy study included 32 real-world plasma specimens with known ctDNA results and eight healthy donor plasmas as known negatives. All 40 specimens generated ctDNA results concordant with expected ctDNA calls (Table 5 of the ESM). Additionally, analysis of the clinical plasma specimens showed that the assay achieved 100% concordance both for variant AFs as low as 0.005% when utilizing 60 ng of cfDNA input and for variant AFs as low as 0.01% when libraries were generated with 10 ng of cfDNA input, which was below the assay's predetermined LOD.

Pooled cfDNA from the eight healthy donors, used as a negative control, generated an expected negative result. Moreover, to test the assay's ability to detect potential sample swaps and contamination, these eight specimens were purposely sequenced with PSPs designed for different people. Results showed that the system was 100% (8/8) successful in identifying mismatches between the specimens tested and the unique SNP signatures of the patients for whom the panels had been generated.

#### 3.2.3 Reproducibility and Precision of ctDNA Detection

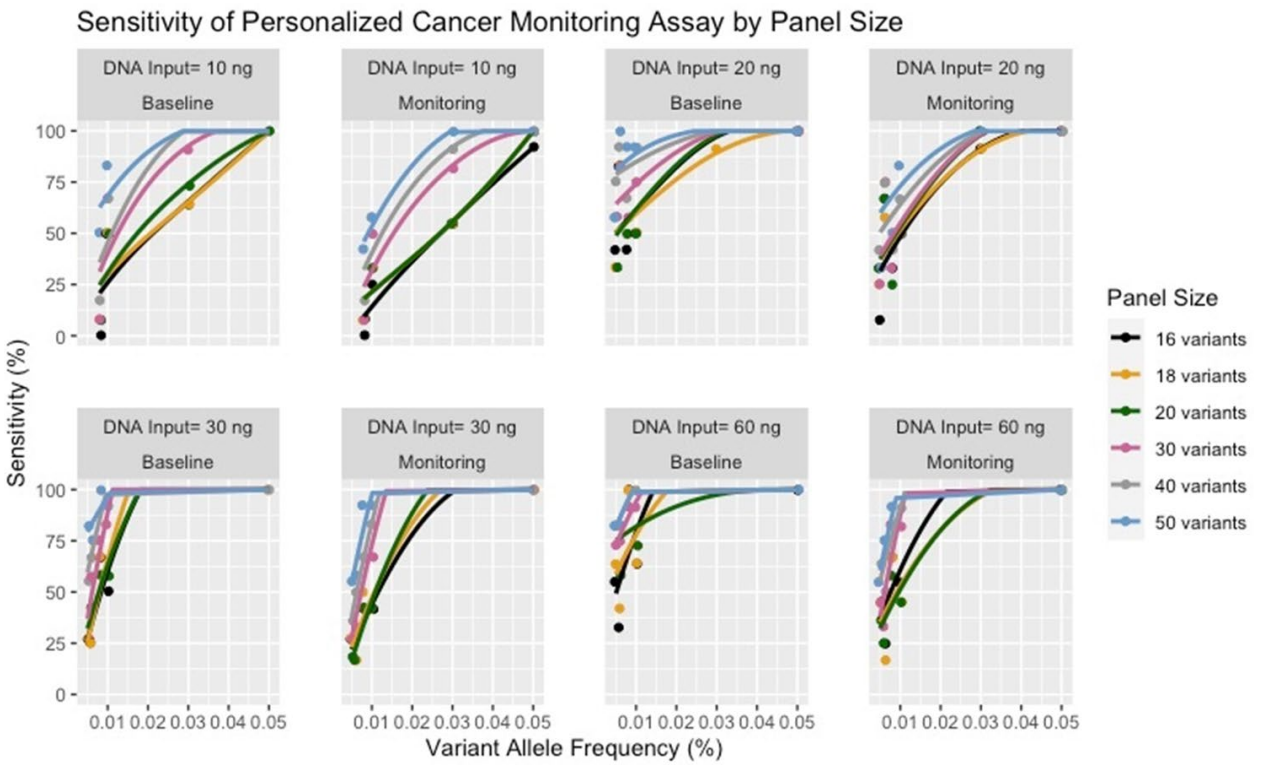
Reproducibility and precision were evaluated using 48 contrived samples from the LOD study featuring variants near the LOD of the assay, with AFs that ranged from 0.003 to 0.1%. To evaluate reproducibility, 108 NGS libraries were prepared in duplicate among different operators, preparation times, and sequencers. To evaluate precision, 36 NGS libraries were prepared in duplicate by a single operator and sequenced on a single run on the same sequencer.

All 144 libraries generated for reproducibility ($n = 108$) and precision ($n = 36$) met QC. The percent agreement for reproducibility and the percent agreement for precision of the assay were 98.2% (95% CI 94.2–99.6) and 97.2% (95% CI 87.7–99.7), respectively (Table 6 of the ESM).

### 3.3 End-to-End Testing

End-to-end testing was performed to validate the entire assay from WES to ctDNA detection using specimens from five patients with stage II or III non-small cell lung cancer (five FFPE tumor specimens, four buffy coats, one FFPE histologically normal tumor-adjacent specimen, and five plasma specimens). The four buffy coats and FFPE histologically normal tumor-adjacent specimen were each matched with one of the five FFPE tumor specimens to evaluate somatic variants in the tumor specimens. The intra-run precision and inter-run reproducibility of WES was evaluated using two input amounts of genomic DNA (200 ng and 500 ng), with WES performed by two operators with two preparation times. Resulting libraries were sequenced on two NovaSeq sequencers in four sequencing runs. A total of 30 tumor WES libraries and 30 matched germline WES libraries were generated (six libraries per specimen with two genomic DNA input amounts in duplicate and one repeat of one input amount in duplicate; Table 5). Patient-specific panel design was then attempted for each of the 30 tumor-germline paired libraries. Patient-specific panels were successfully designed from 24 of the 30 tumor libraries for four specimens; the

**A**




**B**

| 10ng-Baseline | Panel size | | | | | |
|---|---|---|---|---|---|---|
| Variant AF(%) | 16 | 18 | 20 | 30 | 40 | 50 |
| 0.008 | 0% | 8% | 8% | 8% | 17% | 50% |
| 0.01 | 50% | 50% | 50% | 67% | 67% | 83% |
| 0.03 | 64% | 64% | 73% | 91% | 100% | 100% |
| 0.05 | 100% | 100% | 100% | 100% | 100% | 100% |
| 0.07 | 100% | 100% | 100% | 100% | 100% | 100% |
| 0.1 | 100% | 100% | 100% | 100% | 100% | 100% |

| 10ng-Monitoring | Panel size | | | | | |
|---|---|---|---|---|---|---|
| Variant AF(%) | 16 | 18 | 20 | 30 | 40 | 50 |
| 0.008 | 0% | 8% | 8% | 8% | 17% | 42% |
| 0.01 | 25% | 33% | 33% | 50% | 58% | 58% |
| 0.03 | 55% | 55% | 55% | 82% | 91% | 100% |
| 0.05 | 92% | 100% | 100% | 100% | 100% | 100% |
| 0.07 | 100% | 100% | 100% | 100% | 100% | 100% |
| 0.1 | 100% | 100% | 100% | 100% | 100% | 100% |

| 20ng-Baseline | Panel size | | | | | |
|---|---|---|---|---|---|---|
| Variant AF(%) | 16 | 18 | 20 | 30 | 40 | 50 |
| 0.005 | 42% | 33% | 33% | 58% | 75% | 58% |
| 0.006 | 83% | 83% | 83% | 83% | 92% | 100% |
| 0.008 | 42% | 50% | 50% | 58% | 67% | 92% |
| 0.01 | 50% | 50% | 50% | 75% | 92% | 92% |
| 0.03 | 100% | 91% | 100% | 100% | 100% | 100% |
| 0.05 | 100% | 100% | 100% | 100% | 100% | 100% |

| 20ng-Monitoring | Panel size | | | | | |
|---|---|---|---|---|---|---|
| Variant AF(%) | 16 | 18 | 20 | 30 | 40 | 50 |
| 0.005 | 8% | 25% | 33% | 25% | 42% | 33% |
| 0.006 | 67% | 58% | 67% | 75% | 75% | 100% |
| 0.008 | 33% | 42% | 25% | 33% | 42% | 50% |
| 0.01 | 50% | 50% | 50% | 50% | 67% | 83% |
| 0.03 | 91% | 91% | 100% | 100% | 100% | 100% |
| 0.05 | 100% | 100% | 100% | 100% | 100% | 100% |

| 30ng-Baseline | Panel size | | | | | |
|---|---|---|---|---|---|---|
| Variant AF(%) | 16 | 18 | 20 | 30 | 40 | 50 |
| 0.005 | 27% | 27% | 27% | 27% | 55% | 82% |
| 0.006 | 25% | 25% | 42% | 58% | 67% | 75% |
| 0.008 | 67% | 67% | 58% | 75% | 100% | 100% |
| 0.01 | 50% | 58% | 58% | 83% | 92% | 92% |
| 0.05 | 100% | 100% | 100% | 100% | 100% | 100% |

| 30ng-Monitoring | Panel size | | | | | |
|---|---|---|---|---|---|---|
| Variant AF(%) | 16 | 18 | 20 | 30 | 40 | 50 |
| 0.005 | 27% | 27% | 18% | 27% | 36% | 55% |
| 0.006 | 17% | 17% | 17% | 33% | 50% | 58% |
| 0.008 | 42% | 50% | 42% | 67% | 67% | 92% |
| 0.01 | 42% | 42% | 42% | 67% | 83% | 92% |
| 0.05 | 100% | 100% | 100% | 100% | 100% | 100% |

| 60ng-Baseline | Panel size | | | | | |
|---|---|---|---|---|---|---|
| Variant AF(%) | 16 | 18 | 20 | 30 | 40 | 50 |
| 0.005 | 55% | 64% | 82% | 73% | 82% | 82% |
| 0.006 | 33% | 42% | 58% | 75% | 83% | 83% |
| 0.008 | 100% | 100% | 100% | 92% | 100% | 100% |
| 0.01 | 64% | 64% | 73% | 91% | 100% | 100% |
| 0.05 | 100% | 100% | 100% | 100% | 100% | 100% |

| 60ng-Monitoring | Panel size | | | | | |
|---|---|---|---|---|---|---|
| Variant AF(%) | 16 | 18 | 20 | 30 | 40 | 50 |
| 0.005 | 45% | 45% | 36% | 45% | 64% | 55% |
| 0.006 | 25% | 17% | 25% | 33% | 50% | 75% |
| 0.008 | 67% | 67% | 58% | 83% | 83% | 92% |
| 0.01 | 55% | 45% | 45% | 82% | 91% | 100% |
| 0.05 | 100% | 100% | 100% | 100% | 100% | 100% |

**Fig. 4** Assay sensitivity with different panel sizes. Patient-specific panels with 16–50 variants were assessed along with cell-free DNA input amounts (10–60 ng) and analytical thresholds (baseline and monitoring). **A**. The *y*-axis shows sensitivity (%) and the *x*-axis shows variant allele frequency (AF) [%]. Larger panel size, higher input amount, and baseline threshold correlated with detection at lower AFs. **B**. Heat plot of sensitivity data points

remaining six libraries, all from one specimen, did not have a sufficient number of somatic variants to generate PSPs.

Cell-free DNA-derived libraries were then generated using 24 PSPs on cfDNA from four patients using a maximum input of 60 ng and a minimum input of 10 ng. Of the

**Table 4** Observed versus expected ctDNA calls in the study on the accuracy of ctDNA detection

| | Variant allele frequency | | | | | |
|---|---|---|---|---|---|---|
| | 0.005 | 0.008 | 0.01 | 0.05 | 0.1 | 0 |
| cfDNA 10 ng | NA | 9/12 (75.0%) | 11/12 (91.7%) | 12/12 (100.0%) | 12/12 (100.0%) | NA |
| cfDNA 25 ng | NA | 12/12 (100.0%) | 12/12 (100.0%) | 12/12 (100.0%) | NA | 8/8 (100%) |
| cfDNA 60 ng | 8/8 (100.0%) | 8/8 (100.0%) | NA | NA | NA | NA |

*cfDNA* cell-free DNA, *ctDNA* circulating tumor DNA, *NA not applicable*

Results for 108 libraries (both contrived ctDNA-positive samples and negative control samples) are shown by the cfDNA input amount and allele frequency with the baseline threshold

**Table 5** Design of the end-to-end study

| Patient | 200 ng | | 500 ng | | Panel design | No. of attempted panel designs |
|---|---|---|---|---|---|---|
| | No. of replicates | No. of timepoints | No. of replicates | No. of timepoints | | |
| *Whole exome sequencing* | | | | | | |
| 1 | 2 | 1 | 2 | 2 | 1 panel per replicate, at each timepoint and input mass | 6 |
| 2 | 2 | 2 | 2 | 1 | | 6 |
| 3 | 2 | 1 | 2 | 2 | | 6 |
| 4 | 2 | 2 | 2 | 1 | | 6 |
| 5 | 2 | 1 | 2 | 2 | | 6 |

For each of five specimens from patients with non-small cell lung cancer, whole exome sequencing was performed using two input amounts of genomic DNA (200 ng and 500 ng) in duplicate, and two preparation timepoints for one input amount. Six tumor whole exome sequencing libraries and six matched germline whole exome sequencing libraries were generated per patient, and six patient-specific panel designs were attempted per patient

24 PSPs, four could not be used to generate cfDNA libraries because of insufficient amounts of cfDNA; instead, these four PSPs were used on cfDNA from four healthy donor plasma specimens. All 20-resulting plasma NGS libraries passed QC. Analysis of ctDNA demonstrated 100% concordance (20/20) between observed and expected results (Table 7 of the ESM). For the four healthy donor plasma specimens that were processed using non-matched PSPs, the ctDNA-calling algorithm correctly determined that the SNP signatures did not match, highlighting the robustness of the QC measures for catching any sample swaps.

## 4 Discussion

The Invitae Personalized Cancer Monitoring Assay demonstrated robust analytical sensitivity and specificity for both tumor WES and tumor-informed ctDNA detection, including the sensitive detection of ctDNA with cfDNA input as low as 10 ng. With > 99.9% confidence, the LODs ranged from 0.008% AF when using 60 ng of cfDNA input with 18–50 variants and a baseline threshold, to 0.05% AF when using 10 ng of cfDNA input with an 18-variant panel and a monitoring threshold (Figs. 2, 3). Patient-specific panels were successfully generated from all tumor specimens with ≥ 20% tumor content and were designed, though at a lower success rate (80%), from samples with ≥ 10% tumor content. Overall, the results were highly reproducible among operators, preparation times, and sequencers.

We specifically developed our assay to detect low AFs and to discriminate tumor-specific variants from germline variants (including low-level mosaic variants), CHIP variants, and background noise. This, along with the combination of AMP chemistry, UMIs, stringent data QC, and the proprietary ctDNA-calling algorithm likely led to the assay's high sensitivity and specificity for detecting ctDNA at variant AFs as low as 0.008%.

### 4.1 Clinical Utility

Molecular-based MRD testing has become the standard of care for many patients with hematological malignancies as peripheral blood and bone marrow specimens can be repeated collected from patients [20–24]. However,

clinical studies are beginning to show the clinical utility of ctDNA detection in patients with solid tumors as well, with peripheral blood being used as the source of ctDNA. Circulating tumor DNA detection is associated with tumor recurrence within 2 years without therapeutic interventions in many solid tumor types [25–34], and ctDNA levels have been shown to generally correlate with tumor content on imaging [35, 36]. The LOD for tumor detection via imaging is approximately 1.5 cm, or around 110 million cancer cells [37, 38]. Molecular ctDNA detection assays have shown much higher sensitivity and lower LODs than imaging, making earlier detection of MRD or tumor recurrence possible [1, 39]. Research has also shown that ctDNA can be detected before clinical or radiological relapse [1, 26, 33, 40, 41]. Circulating tumor DNA detection has been shown to have lead times over imaging that range from up to 12 months [36, 42] to more than 3 years [18]. Studies have also shown that changes in tumor content have a higher correlation with ctDNA levels than with other molecular markers [43].

The presence of ctDNA has potential clinical implications for risk stratification, treatment monitoring, and post-treatment surveillance. In many cancers, the persistence of ctDNA following resection or treatment can indicate a higher risk of recurrence, and detection of ctDNA may indicate a more aggressive therapy to optimize survival outcomes [18, 26, 36, 44–56]. In addition, ctDNA provides real-time data for monitoring treatment efficacy and detecting drug resistance early, which can be used to establish personalized treatment plans [57–66]. Recurrence may be less likely if ctDNA is not detected, allowing for consideration of treatment de-escalation, which can help patients avoid unnecessary treatment if they are measurably disease free and unlikely to relapse [28, 67]. Moreover, ctDNA can be used for surveillance and longitudinal monitoring for patients in remission or receiving long-term therapy [68].

The CheckMate 816 phase III clinical trial for the assessment of neoadjuvant nivolumab plus chemotherapy in resectable lung cancer utilized this assay to evaluate ctDNA clearance following treatment with nivolumab [17]. Results of the trial identified higher rates of ctDNA clearance in patients treated with nivolumab plus chemotherapy than in those treated with chemotherapy alone, which correlated with longer event-free survival and a pathological complete response [17]. The assay's ability to help assess therapy efficacy opens up endless possibilities for its use in monitoring clinical trial participants.

More recently, TRACERx utilized this assay to detect ctDNA in 197 patients with early-stage lung cancer, longitudinally followed up for up to 5 years. In this study, PSPs with a median of 200 variants (range 70–201) were used per patient, highlighting this assay's flexibility in terms of how many variants can be included in the PSP design [18]. Moreover, because data from ctDNA enabled the determination of intratumor clonality, the study was able to show that patients with polyclonal dissemination had a shorter overall survival than those with monoclonal dissemination, opening a potential new path for the clinical use of ctDNA [18].

Several professional societies and organizations also recognize the clinical utility of ctDNA molecular testing, recommending its use under certain conditions. Recently released guidelines from the American Society of Clinical Oncology recommend the use of ctDNA assays in patients with breast cancer who are candidates for a regimen of phosphatidylinositol 3-kinase inhibitor and hormonal therapy, and guidelines from the European Society for Medical Oncology state that they may be used for selected patients with advanced cancer to direct targeted therapy [69, 70].

### 4.2 Limitations of ctDNA Assays

Circulating tumor DNA assays are prone to biological limitations. Influencing factors include the complex modulation of ctDNA release and the rates at which cfDNA and ctDNA are shed and cleared, which can vary based on factors such as tumor vascularization, temporal variability, and field effects [71]. The amount of ctDNA in the blood depends on many factors, including tumor type, burden, and stage of disease, and may vary over the course of treatment [44, 71–73]. The blood–brain barrier can reduce the amount of ctDNA molecules released into the peripheral blood stream and thereby affect detection rates of ctDNA in blood-based specimens from patients with brain cancer [72]. As such, additional methodologies such as sonobiopsy to increase the ctDNA fraction within the blood or other specimen sources such as cerebrospinal fluid may be needed to detect ctDNA in patients with brain cancer [74–76]. Particular cancer types, such as colorectal cancer, and higher stage tumors have higher shed rates [72, 73, 77–79], and early-stage tumors may have low shed rates that make ctDNA fractions at or below the LOD of a ctDNA assay [72, 80, 81]. Circulating tumor DNA levels generally reflect the overall disease burden of an individual [35, 39], and tumors characterized by lower rates of tumor mutational burden such as mesotheliomas and pilocytic astrocytoma [82, 83] will have fewer targetable somatic variants for a successful PSP design. Current commercial ctDNA assays mainly focus on sequence variants, although methylation patterns and fragmentation profiles have also shown utility in ctDNA assays [84, 85]; moreover, other types of alterations such as fusions and copy number variations warrant future developments. Circulating tumor DNA detection can also be affected by low fractions of ctDNA owing to other factors

such as a patient's body mass index, which can result in ctDNA fractions below an assay's LOD.

The main technical challenges of ctDNA assays are the handling of cfDNA specimens, low signal detection (i.e., typically a very low ctDNA fraction within the specimen, which requires a method with superior sensitivity to successfully detect the signal), and interference from non-somatic variants. The half-life of cfDNA has been estimated to be less than 2 h [86]. This short window of time has been a major challenge for molecular testing, but one that we have mitigated by collecting peripheral blood in cfDNA BCT Streck tubes, in which cfDNA can be stabilized for up to 14 days at 6–37 °C. Proper handling of specimens (i.e., using the right type of tube and following instructions) is critical, as improper handling can lead to a cfDNA yield being too low to generate a result. Even with our assay's superior sensitivity and specificity (≥ 99%) at LODs as low as 0.008% AF, the ctDNA in some tumors will still be below the detection limit. Future developments and technologies may help lower the LODs and amount of cfDNA input required to maintain these same high levels of sensitivity and specificity. Finally, mosaic germline and CHIP variants in peripheral blood specimens have presented challenges for accurate ctDNA detection, as they are unrelated to tumors but difficult to distinguish from somatic variants. If incorporated into PSPs, they can generate false-positive ctDNA results. To overcome this challenge, our assay leverages WES data from both tumor and germline specimens from the same patient for the PSP design, allowing germline, CHIP, and other artifact variants present in the germline specimen to be subtracted from the panels. In addition, our assay incorporates SNP signatures into QC to confirm that tumor, germline, and plasma specimens are all from the same patient, to detect potential sample swaps, and to ensure that no false-positives are reported.

### 4.3 Additional Considerations

As molecular testing options expand, tissue stewardship will be evermore critical. Small biopsies or surgical resections limit the number of molecular tests that can be performed on a single specimen, requiring thoughtful prioritization of molecular tests with the highest clinical utility. Molecular-based, tumor-informed ctDNA testing allows for extensive multi-analyte reporting, including somatic WES for the detection of SNVs, indels, copy number alterations, microsatellite instability, and tumor mutation burden; hereditary cancer germline findings from the germline specimen; and ctDNA results from a serial, minimally invasive peripheral blood collection, all from a single test.

As the clinical utility of ctDNA testing matures, ctDNA analysis and clinical reporting will also evolve. Reporting of quantitative ctDNA values versus qualitative reporting may prove to have stronger clinical utility if the accuracy of these values can be ensured and if they are used within the context of community-wide guidelines for clinical decision making. For longitudinal reporting, clinical reports with prior historical ctDNA values will help oncologists discern the kinetics of ctDNA patterns and tracking of targeted variants over time to assess therapy response, predict prognostic risk, or monitor MRD and tumor recurrence. Additionally, reporting on specific targetable or drug-resistant driver events related to therapeutic modalities from ctDNA testing could enable oncologists to determine optimal treatment for the patient. As with any emerging field, it will be essential to develop clinical practice guidelines along with education and training of clinical personnel for the application and interpretation of ctDNA testing. Harmonization of reported results between different laboratories offering this type of test and recommendations on essential reported values will also be critical.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s40291-023-00670-1.

**Acknowledgements** We thank Aaron Garnett, Paula Roberts, Nicole Williams, Dhruiti Legare, Angela Oliviero, and Laura Hyland for their contributions to the development of the technology.

**Authors' contributions** JZ, JR, KS, MH, EM, ME, IC, and MB contributed to writing up the manuscript. RD, AC, and MM conceived and planned the experiments. EW and WO contributed to sample management. AC, MM, and YZ carried out the experiments. LJ, AO, MW, LH, ER, VJ, and NM contributed to the pipeline development and executions. All authors provided critical feedback and helped shape the manuscript.

## Declarations

**Funding** Funding for the preparation of this article was provided internally by Invitae.

**Conflicts of Interest/Competing Interests** All authors are stockholders of Invitae. Laura Johnson, Mike Washburn, Luke Hartje, Erik Reckase, and Verity Johnson were affiliated with Invitae at the time of the study; they are currently employees at Integrated DNA Technologies, Coralville, IA, USA. Jianhua Zhao, Jacquelyn Reuther, Kaylee Scozzaro, Megan Hawley, Emily Metzger, Matthew Emery, Ingrid Chen, Michelle Barbosa, Alijah O'Connor, Yuhua Zhang, Emily Westheimer, William O'Callaghan, Nirav Malani, Adrian Chesh, Michael Moreau, and Robert Daber are currently employees at Invitae.

**Ethics Approval** Deidentified human specimens were either purchased through commercial vendors or acquired through an inter-laboratory exchange. Studies herein comply with applicable federal, state, and local regulatory requirements.

**Consent to Participate** Not applicable.

**Consent for Publication** Not applicable.

**Availability of Data and Material** The datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

**Code Availability** Not applicable.



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