EXHIBIT 2-B – FINAL INVALIDITY CLAIM CHART FOR U.S. PATENT NO. 11,530,454

References: Forshew, T. et al., *Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA* 4(136):36RA68 (May 2012) ("Forshew")

To the extent Natera asserts that Forshew does not disclose or teach one or more limitations of asserted claims 1, 8, 10, 12, 14, 15, 22, 24, 26 or 28, performing the methods as claimed would have been obvious to a person of ordinary skill in the art at the time of the alleged invention considering Forshew in combination with the knowledge of a POSA and one or more of the following references:

Reference: Gerstung, M. et al., *Reliable detection of subclonal single-nucleotide variants in tumour cell populations.* Nat Commun 3, 811 (May 2012) (including its Supplementary Information ("Gerstung")

Reference: Kukita, Y. et al., *Quantitative Identification of Mutant Alleles Derived from Lung Cancer in Plasma Cell-Free DNA via Anomaly Detection Using Deep Sequencing Data* PLOS ONE 8(11):e81468 (Nov. 13, 2013) ("Kukita")

Reference: Patel, A., et al., *Measurement of Nucleic Acid Variants Using Highly-Multiplexed Error-Suppressed Deep Sequencing*, Patent Application WO 2013/138510 AI (Sept. 19, 2013) ("Patel")

A POSA would have considered the disclosures of the aforementioned prior art because the prior art references all concern the same subject matter, namely amplification and/or sequencing of DNA for the purpose of identifying mutations therein, and particularly detection of rare SNVs in a wild-type background. For example, Forshew teaches that its methods for "noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA." Forshew at Title. As another example. Gerstung teaches methods for "reliable detection of subclonal single-nucleotide variants in tumour cell populations." Gerstung at Title; *see also, e.g.*, *id.* at Abstract (explaining clonal evolution model of tumour growth over time); *see also, e.g.*, *id.* at 7 & Fig. 5 (analyzing possible tumour evolution at different time points). As another example, Kukita teaches methods for "Quantitative identification of Mutant Alleles Derived from Lung Cancer in Plasma Cell-Free DNA via Anomaly Detection Using Deep Sequencing Data." Kukita at Title; *see also, e.g.*, *id.* at Abstract ("A temporal analysis suggested potential applications for disease management and for therapeutic decision making to select epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI)."); *see also, e.g.*, at 5 (describing "Temporal changes of EGFR mutation levels during the disease course"). As yet another example, Patel teaches "measurement of nucleic acid variants using highly-multiplexed error-suppressed deep sequencing" Patel at Title; *see also, e.g.*, *id.*at Abstract ("methods and compositions are disclosed for measuring low-abundance DNA variants from a complex mixture of DNA

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molecules" and "allow for extremely sensitive detection and can distinguish true variants from sequencer misreads and PCR misincorporations.").

A POSA would also be motivated to combine Forshew with Gerstung, Kukita, or Patel in order to achieve improved detection of SNVs by increasing sequencing depth and improving the ability to distinguish true SNVs from sporadic errors, as suggested by Forshew. *See, e.g.,* Forshew at 9 (explaining that Forshew sought to "avoid false positives stemming from PCR errors"), 10 ("[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols"); *See, e.g.,* Gerstung at 1 ("reliable detection of subclonal single-nucleotide variants in tumour cell populations"), 2 ("To overcome these limitations [of sequencing error rates], we employ a comparative sequencing strategy"); *See, e.g.,* Kukita at 1 ("we detected rare mutations using a semiconductor sequencer and a set of anomaly detection criteria based on a statistical model of the read error rate at each error position"); *See, e.g.,* Patel at 1-2 ("Methods as described herein also include detecting and distinguishing true nucleic acid variants from misincorporation errors, sequencing errors, and sample misclassification errors."). A POSA would therefore be motivated to turn to references such as Gerstung, Kukita, or Patel because such references teach methods for detecting ultra-rare variants with higher read depth or fidelity and/or with improved algorithms. For example, Gerstung teaches that its methods are directed to overcome the following limitations of next generation sequencers: "However, single-nucleotide errors resulting from target enrichment, library preparation and base calling are frequent on all current sequencing platforms, and they are difficult to separate from true low-frequency single-nucleotide variants (SNVs). Sequencing error rates vary across genomic sites, often reaching up to 1%, and they challenge accurate calling of SNVs present at frequencies below this rate." Gerstung at 2. As another example, Kukita teaches that its methods are directed to overcoming "the high read error rate of next-generation sequencers." Kukita at Abstract. As yet another example, Patel teaches "a method that is able to detect and quantify rare variant sequences." Patel at 1.

A POSA would have a reasonable expectation of success in combining Forshew with Gerstung, Kukita, or Patel because Forshew discloses that "[w]hen applying TAm-Seq to measure a predefined mutation (as opposed to screening thousands of possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus. False detection is less likely, and criteria for confident mutation detection for a predefined substitution can be less stringent than those described above for de novo mutation identification (Supplementary Methods)." Forshew at 3. Thus, Forshew teaches that detecting known SNVs using a tumor-informed approach allows SNV detection with greater sensitivity. Gerstung, Kukita, and Patel all teach methods capable of detecting pre-defined SNVs that are present at less than or equal to 0.015% of the DNA comprising the locus.

For example, a POSA would be motivated to modify Forshew with Gerstung with a reasonable expectation of success to detect an SNV that is present in less than or equal to 0.015% of the cfDNA because Forshew discloses detecting an SNV if it is present "above the respective substitution-specific background distribution" (Forshew at 3) and discloses substitution-specific background frequences below 0.015% for at least one SNV. *See* Forshew at Figure 1C, Figure 1D. A POSA would have a reasonable expectation of success

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because Forshew discloses performing TAm-Seq on healthy control samples (*see, e.g.,* Forshew at 4) and determining "substitution-specific background frequencies" (*see, e.g.,* Forshew at 3, 4, Forshew supplementary materials at 4). Like Forshew, Gerstung uses a healthy control sample "for estimating the local error rate, which increases the power for calling true variants at a given false-positive rate." Gerstung at 2. Forshew teaches that control samples can be pooled with test samples for sequencing (*see* Forshew at 4), making it easy to analyze a control sample. A POSA would also have a reasonable expectation of success because Gerstung discloses estimating local background error rates allows detection of SNVs present at variant frequencies of 0.0001 or 1 in 10,000 alleles (Gerstung at 2, 4). Furthermore, Gerstung discloses that the power of detecting SNVs present at 0.01% (1e-04) increases with increased read depth. *See* Gerstung supplementary materials at 6, Supplementary Figure S5. Forshew similarly states that higher read depth "could allow for enhanced mutation detection without change to protocols." Forshew at 10. Therefore, a POSA reading Forshew in view of Gerstung would be motivated to perform estimation of local background error rates as taught by Forshew and Gerstung and to increase the sequencing depth in order to detect an SNV present at 0.01% as taught by Gerstung. A POSA would have a reasonable expectation of success for increasing the sequencing depth because in Illumina sequencing experiments "it is very easy to increase the coverage or sequenc[ing] depth" by "simply sequenc[ing] more." *See, e.g.,* Illumina Technical Note: Sequencing (NEOGEN00288666) at 2. For example, Forshew taught in one example that a single lane of sequencing provided a "mean read depth of 3200 above Q30 for each of the 9024 expected read groups (48 amplicons × 2 directions × 94 barcoded samples)." Forshew at 3. A POSA would reasonably expect to increase sequencing depth by dedicating a single lane sequencing lane to fewer different samples (e.g., one control and one test sample, rather than 94 different pooled samples) and/or by dedicating one or more sequencing lanes to a given sample. A POSA would also be motivated to combine Forshew and Gerstung and would have a reasonable expectation of success because Gerstung used the same sequencing instrument as Forshew (an Illumina GAIIx sequencer) (Gerstung at 2, 7, 8, Gerstung Supplementary Materials at 16, 18) and showed that sequencing errors occurred at rates well below 0.015%. *See, e.g.,* Gerstung at 2, 4, Figure 2.

As another example, a POSA would be motivated to modify Forshew with Kukita with a reasonable expectation of success to detect an SNV that is present in less than or equal to 0.015% of the cfDNA because Forshew discloses detecting an SNV if it is present "above the respective substitution-specific background distribution comprising the sequence reads" (Forshew at 3) and discloses substitution-specific background frequences below 0.015% for at least one SNV. *See* Forshew at Figure 1C, Figure 1D. A POSA would have a reasonable expectation of success because Forshew discloses performing TAm-Seq on healthy control samples (*see, e.g.,* Forshew at 4) and determining "substitution-specific background frequencies" (*see, e.g.,* Forshew at 3, 4, Forshew supplementary materials at 4). Like Forshew, Kukita performs sequencing of a sample from a normal individual to determine local background error rates (a "read error rate" (RER)). *See* Kukita at 2. As discussed above, Forshew teaches that control samples can be pooled with test samples for sequencing (*see* Forshew at 4), making it easy to analyze a control sample. A POSA would also have a reasonable expectation of success because Kukita discloses that "With an increasing read depth and decreasing RER, the detection limit

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decreases" and that a detection limit of 0.01% (1 in 10,000) can be achieved with 100,000 reads when the RER is below 0.01%. Kukita at 2. Forshew similarly states that higher read depth "could allow for enhanced mutation detection without change to protocols." Forshew at 10. Therefore, a POSA reading Forshew in view of Kukita would be motivated to perform estimation of local background error rates as taught by Forshew and Kukita and to increase the sequencing depth in order to detect an SNV present at 0.01% as taught by Kukita. A POSA would have a reasonable expectation of success for increasing the sequencing depth because Forshew used an Illumina sequencer (Forshew at 3), and in Illumina sequencing experiments "it is very easy to increase the coverage or sequencing depth" by "simply sequenc[ing] more." *See, e.g.,* Illumina Technical Note: Sequencing. For example, Forshew taught in one example that a single lane of sequencing provided a "mean read depth of 3200 above Q30 for each of the 9024 expected read groups (48 amplicons × 2 directions × 94 barcoded samples)." Forshew at 3. A POSA would reasonably expect to increase sequencing depth by dedicating a single lane sequencing lane to fewer different samples (e.g., one control and one test sample, rather than 94 different pooled samples) and/or by dedicating one or more sequencing lanes to a given sample. Alternatively, a POSA would have reasonable expectation of success performing TAm-Seq as taught by Forshew with a PGM Torrent sequencer as taught by Kukita, because Kukita teaches local RERs below 0.01% and sequencing depths of greater than 100,000 for multiple target loci using a PGM torrent sequencer (Kukita at 2-3).

As another example, a POSA would be motivated to modify Forshew with Kukita with a reasonable expectation of success to use "a set of anomaly detection criteria based on a statistical model of the read error rate at each error position," (Kukita at Abstract) and "the statistical model of the RER can be constructed from sequence data from the target regions of a sufficient number of normal individuals carrying no mutations." Kukita at 2. Modifying Forshew with Kukita to involve such statistical model could overcome "the major obstacle [of] high read error rate of next-generation sequencers" and achieve "the detection of rare mutants using next generation sequencing." Kukita at Abstract.

A POSA would be motivated to combine Forshew with Gerstung or Kukita would have a reasonable expectation of success because the references teach a similar approach to SNV detection. For example, Forshew teaches that "[t]he minimal nonreference AFs that could be detected depend on the read depth and background rates of nonreference reads, which vary per locus and substitution type." Forshew at 3. Gerstung also teaches an approach that involves "estimating the local error rate, which increases the power for calling true variants at a given false-positive rate." Gerstung at 2. Similarly, Kukita also teaches an approach to "detect[] rare mutations using a semiconductor sequencer and a set of anomaly detection criteria based on a statistical model of the read error rate at each error position" that "would increase the accuracy of high error rate position, [] sensitivity and specificity." Kukita at Abstract and 7. A POSA would also be motivated to combine Forshew with Gerstung or Kukita because Forshew states that higher read depth would improve sensitivity, and both Gerstung and Kukita specifically show that increasing read depth together with the method of estimating local error rates allows detection of SNVs present at frequencies less than 0.015% VAF (e.g., SNVs present at 0.01% VAF). *See*

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Gerstung Supplementary Materials at Supplementary Fig. S5B; Kukita at 2 and Figure 1; *see also* "General Knowledge of a POSA" section of NeoGenomics' Preliminary Invalidity Contentions cover pleading (discussing sequencing depth and SNV detection from sequencing reads).

Like Forshew, Gerstung discloses that "additional sensitivity for calling low-frequency variants can be gained by increasing sequencing depth." *See* Gerstung at 2, Gerstung Supplementary Materials at Supplementary Fig. S5B. Gerstung further explains that "[r]oughly, the required coverage for calling a variant needs to be at least ten times higher than its inverse frequency. For large genomes, the power of SNV calling is diminished by multiple testing corrections, but it remains high for variants present in 1/1,000 alleles." Gerstung at 2, Gerstung Supplementary Materials at Supplementary Fig. S5C. A POSA would further have a reasonable expectation of success because references such as Gerstung achieved detection of a SNV present at frequencies of 0.0001 or 1/10,000 alleles (*see, e.g.,* Gerstung at Table 2, Abstract, 4) using only 100 ng of input DNA as the template for PCR (*see* Gerstung at 8). *See also* Kukita at Figure 1a (improved sensitivity by increasing sequencing depth). Furthermore, a POSA would have a reasonable expectation of success because methods for multiplex amplification, including multiplex amplification of more than 10 target loci in a single reaction, were well known in the art as of Natera's asserted priority date, as explained in the "General Knowledge of a POSA" section of NeoGenomics' Preliminary Invalidity Contentions cover pleading. Finally, Gerstung and Kukita provide the exact solution suggested by Forshew, including "[h]igher read depth" and "improved algorithms" which allow for "enhanced mutation detection without change to protocols"—that is, combining Gerstung or Kukita with Forshew would not require any modifications to the TAm-Seq multiplex PCR protocol.

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Claim	**Forshew**
1[pre]. A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV)	To the extent the preamble is construed to be limiting, Forshew discloses a method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample. *See, e.g.,* Forshew at Abstract ("Plasma of cancer patients contains cell-free tumor DNA that carries information on tumor mutations and tumor burden" and demonstrates "use of [tagged-amplicon deep sequencing (TAm-Seq)] to noninvasively identify the origin of metastatic relapse in a patient with multiple primary tumors."); Forshew at 9 ("Whole-genome sequencing of tumor material was used to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy. Ten mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci . . . Serial plasma samples were

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mutations in the plasma sample, the method comprising:	collected over the course of 497 days, both before and after treatment. We performed TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and tracked dynamics of all mutations in parallel."); Forshew at 4 ("We applied TAm-Seq to directly identify mutations in plasma of cancer patients."); Forshew supplemental materials at 1 ("Dilution of plasma DNA samples to determine allele quantification accuracy"); Forshew supplemental materials at 17–19, Tables S6–S7 (identification of tumor mutations that are single nucleotide changes or substitutions in a genome, *i.e.*, SNVs).
1[a]. performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;	Forshew discloses performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations. *See, e.g.,* Forshew at 3, 9, 10, Table S3, Table S7. *See, e.g.,* Forshew at 9 (performing "[w]hole-genome sequencing of tumor material [] to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy."); Forshew supplemental materials at 19, Table S7 (identifying mutations that are single nucleotide changes (*i.e.*, SNVs)); Forshew at 3 (disclosing the use of "TAm-Seq to sequence DNA extracted from 47 formalin-fixed, paraffin-embedded (FFPE) tumor specimens of ovarian cancers (table S3)" and that these samples "were also sequenced for TP53 by Sanger sequencing"). Because TP53 is a tumor suppressor gene associated with cancer, a POSA would have understood that Sanger sequencing to identify mutations in TP53 constitutes identification of tumor-specific SNV mutations. Forshew further discloses that "43 single-base substitutions were called (table S3)" and "[t]hese matched 100% of mutations identified by Sanger sequencing and included three additional mutations at low [allele frequencies] that were below detection thresholds of Sanger sequencing (fig. S2)." Forshew at 3. Forshew identifies 40 single-base substitutions (i.e., SNVs) in the ovarian tumor sample. Forshew supplemental materials at 13–14, Table S3. These "single-base substitutions" are tumor-specific SNVs, and a POSA would have understood that such tumor-specific SNVs could be identified in the same manner by performing whole exome sequencing or whole genome sequencing because the whole exome sequences and whole genome sequences include the SNVs.

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Claim	**Forshew**
	As another example, Forshew teaches Table S7:

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Claim	**Forshew**

Table S7: Mutations and amplicons studied in one breast cancer patient. To identify multiple mutations, genomic libraries from tumour and matched normal tissues were prepared using the standard Illumina paired-end sample preparation kit according to the manufacturer's instructions. DNA fragments of 300 bp were sequenced using paired-end 100-bp reads on an Illumina HiSeq 2000 sequencer to 30× genomic coverage. Sequencing reads were aligned to the hg19 reference genome using ELAND (Illumina) and subsequent data analysis was performed using the CASAVA 1.8 analysis pipeline (Illumina) to identify somatic mutations. We selected 10 mutations and designed an amplicon of <120 bp to cover each mutation. We validated these mutations by TAm-Seq in the tumour material and then followed them in serial plasma samples collected from the same patient.

Gene	Genomic location of amplicon (genome build hg19)	Genomic location of mutation (genome build hg19)	Reference allele	Mutant allele
CD1A	chr1:158226710-158226810	chr1:158226762	C	T
BUB1	chr2:111430288-111430374	chr2:111430343	G	A
IQCA1	chr2:237402413-237402499	chr2:237402439	G	A
PIK3CA	chr3:178936048-178936130	chr3:178936091	G	A
ARAP3	chr5:141041694-141041773	chr5:141041739	G	A
MET	chr7:116398593-116398675	chr7:116398635	C	T
GIMAP5	chr7:150439702-150439805	chr7:150439730	C	T
ZFYVE21	chr14:104195437-104195539	chr14:104195486	G	A
KIAA0406	chr20:36641162-36641252	chr20:36641201	C	T
ADAMTS1	chr21:28214239-28214357	chr21:28214274	C	G

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Claim	**Forshew**
	As another example, Forshew teaches "We designed a set of 48 primer pairs to amplify 5995 bases of genomic sequence covering coding regions (exons and exon junctions) of TP53 and PTEN, and selected regions in EGFR, BRAF, KRAS, and PIK3CA (table S1) by overlapping short amplicons (Fig. 1A). The sequenced regions cover mutations that account for 38% of all point mutation in the COSMIC database (v55) (32)." Forshew at 3. As another example, Forshew discloses: "Whole-genome sequencing of tumor material was used to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy. Ten mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci (table S7). Serial plasma samples were collected over the course of 497 days, both before and after treatment." Forshew at 9.
1[b]. performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in	Forshew discloses multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume. *See, e.g.,* Forshew at 9 (using "[w]hole-genome sequencing of tumor material . . . to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy," wherein "[t]en mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci (table S7)" and performing targeted multiplex amplification that amplifies the ten different tumor-specific SNV mutations in cfDNA from a plasma sample by performing "TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and track[ing] dynamics of all mutations in parallel"); Forshew at 3 ("We therefore applied a two-step amplification process: a limited-cycle preamplification step where all primer sets were used together to capture the starting molecules present in the template, followed by individual amplification to purify and select for intended targets. (Fig. 1B) (Supplementary Methods)."); Forshew supplemental materials at 2 ("Preamplification reactions were carried out in 10-µl reaction volumes containing 50 nM of each forward and reverse target-specific primer . . . Reactions were subjected to 15 cycles of amplification."); Forshew at 2, Fig. 1B (depicting the targeted multiplex amplification, wherein "[m]ultiple regions were amplified in parallel. An initial preamplification step was performed for 15 cycles using a pool of the target-specific primer pairs").

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Claim	**Forshew**
the same reaction volume; and	Forshew further discloses that "[p]lasma of cancer patients contains cell-free tumor DNA that carries information on tumor mutations and tumor burden" and teaches the use of TAm-Seq to monitor cfDNA. Forshew at Abstract; *see also* Forshew at 9 ("Serial plasma samples were collected over the course of 497 days, both before and after treatment. We performed TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and tracked dynamics of all mutations in parallel."). Forshew discloses amplicons that fall within the recited range of 50–150 bases. *See* Forshew at 9 ("[S]hort amplicons (<120 bp) were designed to cover the mutation loci."); Forshew supplemental materials at 19, Table S7 (listing genomic locations of amplicons with a start location and a stop location, thus demonstrating amplicons with lengths of more than 50 bases and less than 120 bases). Forshew also discloses performing TAm-Seq with a degree of multiplex greater than ten. *See* Forshew Fig 1A, Forshew supplemental materials at 9, Table S1 (disclosing performing TAm-Seq with 48 sequence-specific primer pairs to amplify 5995 bases of genomic sequence). Forshew discloses performing multiplex amplification with a combination of "each forward and reverse target-specific primer" in the same volume. Forshew supplemental materials at 2; *see also id.* at 17-18 (Table S6); Forshew at 6 (Table 2).
1[c]. sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads,	The Court construed the term "sequencing the amplicons" to have its plain and ordinary meaning. Doc. 280 at 10. The Court construed the term "sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads" to require that the detecting and sequencing are part of the same step. *Id.* at 11. In addition, Natera has failed to supplement its response to NeoGenomics' Interrogatory No. 14 to properly address NeoGenomics' Initial Invalidity Contentions served on February 14, 2024 and the Court's Claim Construction Order issued on May 15, 2024 (Doc. 280). NeoGenomics reserves the right to supplement its contentions to the extent Natera supplements its response to Interrogatory No. 14 or there are any modifications to the Court's claim construction ruling.

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Claim	**Forshew**
	Under Natera's apparent interpretation of the Court's adopted construction, Forshew discloses sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads. *See, e.g.,* Forshew at 2, Fig. 1B; Forshew at 3 (pooling and sequencing DNA that has been amplified through the TAm-Seq method and explaining that "[w]e performed 100-base single-end sequencing (GAIIx sequencer, Illumina), with an additional 10 cycles using the barcode sequencing primer, generating ~30 million reads per lane."); Forshew at 9 ("[W]e demonstrate noninvasive identification of mutant alleles in plasma, at AFs as low as 2%, by targeted deep sequencing of circulating DNA."). Forshew further discloses analyzing the sequence data to detect tumor-specific SNV mutations by using a bioinformatics pipeline. *See* Forshew supplemental materials at 4 ("De-multiplexing and alignment," "Mutation identification," and "Mutation detection"); *see also* Forshew at 5, Table 1 (identifying tumor-specific SNV mutations in plasma samples from seven ovarian cancer patients); Forshew supplemental materials at 19, Table S7 ("[S]ubsequent data analysis was performed using the CASAVA 1.8 analysis pipeline (Illumina) to identify somatic mutations."). The Court's Claim Construction Order states that "NeoGenomics is correct that the sequencing must be done on the amplicons obtained from the targeted multiplex amplification, not on some other set of amplicons obtained from some other source or process; the term "the" must mean something. Doc. 280 at 8. To the extent to the Court's construction is interpreted to require sequencing amplicons from the multiplex PCR step rather than sequencing only a subset of the amplicons from the multiplex PCR step, such a modification of Forshew would have been obvious to a person of ordinary skill in the art. Forshew teaches that the multiplex PCR step was designed to avoid unwanted side products. *See* Forshew at 3 ("The final concentration of each primer in the preamplification reaction was 50 nM, reducing the potential for interprimer interactions, and 15 cycles of long-extension (4 min) polymerase chain reaction (PCR) were used to remain in the exponential phase of amplification."); Forshew supplemental materials at 2 ("Primer designs were screened against the hg19 reference genome to prevent cross-product generation, and were screened to prevent primer-dimer interactions and formation of intramolecular secondary structure."). Accordingly, a POSA would have understood that it was not necessary to perform single-plex PCR to improve purity.

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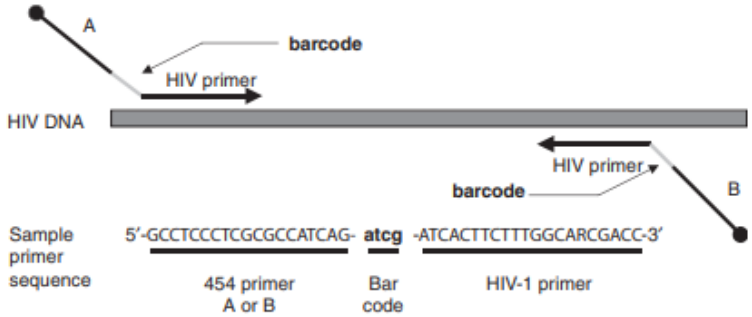
Claim	**Forshew**
	Further, it would have been obvious to a POSA to incorporate sample barcodes and sequencing adapters into the sequence-specific primers used in the targeted multiplex amplification such that they would be incorporated into the amplicons resulting from the multiplex amplification step. A POSA would have been motivated to do so because omitting the single-plex PCR step would simplify the overall process by requiring only a single amplification step. Additionally, a POSA would have had a reasonable expectation of success for two reasons. First, Forshew already teaches multiplex PCR conditions that avoid unwanted side products and preserve relative abundance of mutations, such that a single-plex PCR used to enhance purity would not be required. Second, it is well-established in the art that targeted amplification using a single amplification reaction step can be used to attach sequencing platform-specific barcodes prior to sequencing. *See, e.g.,* Christian Hoffman et al., *DNA bar coding and pyrosequencing to identify rare HIV drug resistance mutations*, 35 Nucleic Acids Research, 13:1-8 at 3 (June 18, 2007) (teaching that "[g]enomic DNA samples of interest are amplified using primers that include 5' extensions providing binding sites for the 454A and B primers" that are required for sequencing); *id.* at 4, Figure 2 (showing the single amplification step, reproduced below).  **Figure 2.** The DNA bar coding system. Primers are shown diagrammatically, illustrating the placement of the DNA bar code between the 454 primers (**A** and **B**) and the HIV-complementary sequences. An example of a primer sequence is shown at the bottom. DNA 5′ ends in the primers are shown as the black balls. To the extent Natera argues claim 1 excludes a single plex amplification step between step 1(c)[1] and 1(c)[2], performing Forshew's method without the single-plex amplification step would be an obvious modification.

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Claim	**Forshew**
	First, Natera has admitted that multiplex PCR amplifications of up to 50 or 100 targets were known in the art. *See, e.g.,* "General Knowledge of a POSA" section in NeoGenomics' Final Invalidity Contentions; U.S. Patent Application Publication 2012/0270212 at [0217] ("the prior art includes disclosures of successful multiplexed PCR reactions involving pools of up to about 50 or 100 primers, and not more"). It would have been obvious to modify Forshew to use any of the other known methods to avoid primer dimer formation during multiplex PCR (e.g., primer design to avoid primer dimers or nested multiplex PCR) instead of an intervening single-plex PCR. *See* "General Knowledge of a POSA" section in NeoGenomics' Final Invalidity Contentions. A POSA would be motivated to use such primer design tools or nested multiplex PCR because such methods allow all amplification steps to be performed in a single tube. *See id.* To the extent Natera contends the claims allow an intervening step to remove spurious amplicons generated in the multiplex amplification before sequencing, such techniques were also known in the art (*e.g.*, automated gel extraction) and would be an obvious modification of Forshew. *Id.* A POSA would be motivated to modify Forshew to use an automated gel extraction method instead of an intervening single-plex PCR because of the advantages of automated gel extraction for removing spurious products such as primer dimers while eliminating variability and cross-contamination, requiring minimal hands-on time, and producing high yield. *See, e.g.*, *id.*; Thermo Fisher Scientific Inc. *Pippin™ DNA size selection systems: Improving NGS Data Everywhere* (2013).
1[d]. wherein the sequencing has a depth of read of at least 50,000 per target locus.	Forshew discloses sequencing with a depth of read of at least 50,000 per target locus. Forshew discloses a sequencing depth of at least 15,400, and an additional depth of at least 50,000 would have been obvious to a POSA in view of the express teaching of Forshew, *e.g.*, because higher read depth could allow for enhanced detection of lower levels of mutation without change to protocols. *See* Forshew at 10. A read depth of 50,000 would also be obvious because Forshew discloses background error rates well below 0.015%, and a POSA would understand that more sensitive SNV detection would be achieved by higher read depths at such low error rates or with greater tolerance for false positives (e.g., accepting lower confidence). *See, e.g.*, Forshew at 3, Table S3.

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	For example, Forshew acknowledges that a "current limitation of TAm-Seq" was its "detection limit compared to assays that target individual loci" which were able "to detect two to three order of magnitude lower frequencies." Forshew at 10; *see also* Forshew at 9. Forshew teaches that higher read depth can overcome this limitation: "[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols." Forshew at 10. In addition to Forshew's express teaching of a higher read depth, a POSA would have understood that using "additional replicates" is also using "higher read depth" because depth of read is a measure of the number of times a particular sequence is read or sequence reads that map to a given genetic locus, and one can increase the number of such reads by using "additional replicates." For example, Forshew teaches: "Sequencing adaptors were separately attached at either end and the products mixed together, such that single-end sequencing generated separate sets of forward and reverse reads. We performed 100-base single-end sequencing (GAIIx sequencer, Illumina), with an additional 10 cycles using the barcode sequencing primer, generating ~30 million reads per lane. This produced an average read depth of 3250 for each of 96 barcoded samples for 48 amplicons read in two possible orientations." Forshew at 3. The read depth for a single replicate of a given locus would therefore be 6,500 per replicate, or 13,000 across the two replicates used for TAm-Seq. *See* Forshew at 3, 9, 7, FIG. 4D legend ("TAm-Seq results are reported as the mean frequency of duplicate analyses"), 4, FIG. 2 legend ("measured mutant AF (average of both repeats)"), Forshew Supplementary Information at 4, 17, 18. Accordingly, Forshew teaches both the motivation and reasonable expectation of success to increase the read depth to at least 50,000 per target locus. Further evidence of motivation and reasonable expectation of success to increase the read depth to at least 50,000 per target locus is found in NEOGEN00275278–NEOGEN00275334 and NEOGEN00275270–NEOGEN00275277. *See, e.g.*, NEOGEN00275326 (describing tables plotting the "number of reads for each barcode according to which block of 48 barcodes it belongs to") and NEOGEN00275329 (providing read counts of greater than 50,000 for some loci (e.g., in columns labeled FLD0200, FLD0217, FLD0233, FLD0234, FLD0235, FLD0236, FLD0237, FLD0238, and FLD0239).

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Claim	**Forshew**
	Kukita teaches the sequencing has a depth of read of at least 50,000 per target locus. *See, e.g*. Kukita at 2-6 and 8. For example, Kukita teaches "Semiconductor sequencing. Sequencing template preparation (emulsion PCR and beads-enrichment) from sequencing libraries was carried out using an Ion OneTouch Template Kit (Life Technologies) and Ion OneTouch system (Ion OneTouch Instrument and Ion OneTouch ES, Life Technologies) according to manufacturer's protocol. Prepared templates were sequenced using Ion Sequencing Kit v2 and the Personal Genome Machine (Life Technologies). Number of nucleotide flows during sequencing was set to 200 (50 cycles). Torrent Suite 2.2 (Life Technologies) was used for converting raw signals into base calls, and extracting FASTQ files of sequencing reads. Read depth for one assay mostly exceeded 100,000. Sequencing data were deposited in DDBJ Sequence Read Archive (accession number: DRA001029)." Kukita at 8. *See also* Gerstung at 2, Supplemental Figure S5. For example, Gerstung discloses that "[p]ower calculations show that additional sensitivity for calling low-frequency variants can be gained by increased sequencing depth." Gerstung at 2. Further, Gerstung supplementary figure S5B shows that increasing read depth from 10,000 to 100,000 improves the significant fraction of variants detected at frequencies of 1e-04 (0.0001 or 1 in 10,000). Gerstung Supplementary Materials at Supplementary Figure S5B. *See, e.g.,* Patel at 72 ("The total number of raw clonal paired-end sequences was 20,511,389. After application of the various filters described above, the remaining sequences numbered 11,184,975. The 40 different gene target regions were fairly evenly represented among the filtered sequences. The median sequence count for the 40 gene-specific regions (all barcodes) was 166,867.").
8. The method of claim 1, wherein the targeted multiplex amplification	Forshew discloses that the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation.

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amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation.	*See, e.g.,* Forshew at 2 (performing targeted multiplex amplification to amplify 20 and 48 target loci); Forshew supplemental materials at 9–10, Table S1 (performing TAm-Seq with 48 sequence-specific primer pairs to amplify 5995 bases of genomic sequence covering coding regions of *TP53* and *PTEN*, as well as selected regions in *EGFR*, *BRAF*, *KRAS*, and *PIK3CA* by overlapping short amplicons); Forshew at 6 (Table 2); Forshew supplemental materials at 17-18 (Table S6); Forshew at 10 (disclosing that more loci would "increase [] the robustness for tumor monitoring by compensating for effects of sampling noise or mutational drift"). *See, e.g.,* Forshew at 9 (using "[w]hole-genome sequencing of tumor material . . . to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy," wherein "[t]en mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci (table S7)" and performing targeted multiplex amplification that amplifies the ten different tumor-specific SNV mutations in cfDNA from a plasma sample by performing "TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and track[ing] dynamics of all mutations in parallel"); Forshew at 3 ("We therefore applied a two-step amplification process: a limited-cycle preamplification step where all primer sets were used together to capture the starting molecules present in the template, followed by individual amplification to purify and select for intended targets. (Fig. 1B) (Supplementary Methods)."); Forshew supplemental materials at 2 ("Preamplification reactions were carried out in 10-µl reaction volumes containing 50 nM of each forward and reverse target-specific primer . . . Reactions were subjected to 15 cycles of amplification."); Forshew at 2, Fig. 1B (depicting the targeted multiplex amplification, wherein "[m]ultiple regions were amplified in parallel. An initial preamplification step was performed for 15 cycles using a pool of the target-specific primer pairs"). Forshew further discloses that "[p]lasma of cancer patients contains cell-free tumor DNA that carries information on tumor mutations and tumor burden" and teaches the use of TAm-Seq to monitor cfDNA. Forshew at Abstract; *see also* Forshew at 9 ("Serial plasma samples were collected over the course of 497 days, both before and after treatment. We performed TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and tracked dynamics of all mutations in parallel."). Forshew also discloses performing TAm-Seq with a degree of multiplex greater than ten. *See* Forshew Fig 1A, Forshew supplemental materials at 9, Table S1 (disclosing performing TAm-Seq with 48 sequence-specific primer pairs to amplify 5995 bases of genomic sequence).

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Claim	**Forshew**
	Forshew discloses performing multiplex amplification with a combination of "each forward and reverse target-specific primer" in the same volume. Forshew supplemental materials at 2; *see also id.* at 17-18 (Table S6); Forshew at 6 (Table 2). In addition, the related '035 patent admits a POSA's "general belief in the art is that multiplexing PCR for sequencing is limited to about 100 assays in the same well." '035 patent at 48:25-29. Thus, the related '035 patent admits that a POSA would have been aware of how to perform multiplex PCR up to 100 assays in a single well, and would have been able to do so as a matter of routine. To the extent Natera asserts that Forshew does not disclose this limitation, a POSA would be motivated to modify Forshew to perform multiplex amplification to amplify 20 to 50 target loci in order to detect additional tumor-specific SNVs without requiring additional reactions. A POSA would have a reasonable expectation of success because Forshew demonstrated successful multiplex amplification of 20 to 50 different loci from cfDNA in a single reaction, and Rabinowitz et al., United States Patent Application Publication No. 2012/0270212 (published Oct. 25, 2012) ("Rabinowitz") disclosed that multiplexed PCR reactions involving pools of up to about 50 or 100 primers were conventional as of Natera's asserted priority date. Rabinowitz at [0217] ("Note that the prior art includes disclosures of successful multiplexed PCR reactions involving pools of up to about 50 or 100 primers, and not more"); [0284] (describing application to cancer patient plasma samples).
10. The method of claim 1, wherein the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations	Forshew discloses that the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample. *See, e.g.,* Forshew supplemental materials at 2 ("Target-specific primers were designed with universal primer sequences (termed CS1 and CS2) appended at the 5'-end (table S1)."); Forshew at 2, Fig. 1 ("Primers were designed to amplify regions of interest in overlapping short amplicons."); Forshew supplemental materials at 2 (Primer design for TAm-Seq); Forshew at 2 (Figure 1B showing the "initial preamplification step was performed for 15 cycles using a poos of target-specific primer pairs.").

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Claim	**Forshew**
identified in the tumor sample.	
12. The method of claim 1, wherein the method further comprises detecting recurrence and/or metastases of the cancer from the SNV mutations detected in the cell-free DNA.	Forshew discloses that the method further comprises detecting recurrence and/or metastases of the cancer from the SNV mutations detected in the cell-free DNA. *See, e.g.,* Forshew at Abstract ("We demonstrated use of TAm-Seq to noninvasively identify the origin of metastatic relapse in a patient with multiple primary tumors . . . We further used TAm-Seq to monitor tumor dynamics, and tracked 10 concomitant mutations in plasma of a metastatic breast cancer patient over 16 months."); Forshew at 4 ("In one plasma sample collected from an ovarian cancer patient at relapse, we also identified a de novo mutation in the tyrosine kinase domain of *EGFR* (exon 21), at AF of 6% (patient 27, Table 1)."); Forshew at 10 ("a method that measures multiple mutations in parallel [when looking for recurrent or residual tumor] can have a low likelihood of a false-negative result even if the detection rate for each mutation is less than 50%.").
14[pre]. A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample, the method comprising:	To the extent the preamble is construed to be limiting, Forshew discloses a method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample, the method comprising. *See* claim 1[pre] above.
14[a]. performing whole exome	Forshew discloses performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations.

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sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;	*See* claim 1[a] above.
14[b]. performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons, wherein the target loci are amplified together in the same reaction volume; and	Forshew discloses performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons, wherein the target loci are amplified together in the same reaction volume. *See* claim 1[b] above.
14[c]. sequencing the amplicons to obtain sequence reads, and	Forshew discloses sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads.

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Claim	**Forshew**
detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads,	The Court construed the term "sequencing the amplicons" to have its plain and ordinary meaning. Doc. 280 at 10. The Court construed the term "sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads" to require that the detecting and sequencing are part of the same step. *Id.* at 11. In addition, Natera has failed to supplement its response to NeoGenomics' Interrogatory No. 14 to properly address NeoGenomics' Initial Invalidity Contentions served on February 14, 2024 and the Court's Claim Construction Order issued on May 15, 2024 (Doc. 280). NeoGenomics reserves the right to supplement its contentions to the extent Natera supplements its response to Interrogatory No. 14 or there are any modifications to the Court's claim construction ruling. Under Natera's apparent interpretation of the Court's adopted construction, Forshew discloses sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads. To the extent Natera argues claim 14 excludes a single plex amplification step between step 14(c)[1] and 14(c)[2], performing Forshew's method without the single-plex amplification step would be an obvious modification. First, Natera has admitted that multiplex PCR amplifications of up to 50 or 100 targets were known in the art. *See, e.g.,* "General Knowledge of a POSA" section in NeoGenomics' Final Invalidity Contentions; U.S. Patent Application Publication 2012/0270212 at [0217] ("the prior art includes disclosures of successful multiplexed PCR reactions involving pools of up to about 50 or 100 primers, and not more"). It would have been obvious to modify Forshew to use any of the other known methods to avoid primer dimer formation during multiplex PCR (e.g., primer design to avoid primer dimers or nested multiplex PCR) instead of an intervening single-plex PCR. *See* "General Knowledge of a POSA" section in NeoGenomics' Final Invalidity Contentions. A POSA would be motivated to use such primer design tools or nested multiplex PCR because such methods allow all amplification steps to be performed in a single tube. *See id.* To the extent Natera contends the claims allow an intervening step to remove spurious amplicons generated in the multiplex amplification before sequencing, such techniques were also known in the art (*e.g.*, automated gel extraction) and would be an obvious modification of Forshew. *Id.* A POSA would be motivated to modify

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Claim	**Forshew**
	Forshew to use an automated gel extraction method instead of an intervening single-plex PCR because of the advantages of automated gel extraction for removing spurious products such as primer dimers while eliminating variability and cross-contamination, requiring minimal hands-on time, and producing high yield. *See, e.g.*, *id.*; Thermo Fisher Scientific Inc. *Pippin™ DNA size selection systems: Improving NGS Data Everywhere* (2013). *See* claim 1[c] above.
14[d]. wherein the method is capable of detecting an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus.	Forshew discloses a method capable of detecting an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus. *See* claim 1[d] above. *See, e.g.,* Forshew at 3 ("Background frequencies of nonreference reads were ~0.1% (median, 0.03%; mean, 0.2%; in keeping with Q30 quality threshold applied)"); Forshew supplemental materials at 7, Fig. S3 (showing frequencies that could be detected with different confidence levels about the background read rate, including frequencies of 0.015% and below); Forshew at 9 ("Enhanced detection down to 1 mutant allele in 10,000 or more wild-type alleles can be obtained."); Forshew supplemental materials at 17-19, Tables S6—S7 (identification of tumor mutations that are single nucleotide changes or substitutions in a genome, *i.e.,* SNVs). Under Natera's interpretation of the claim, Forshew anticipates and/or renders obvious, alone or in combination with other references, asserted claim 14 of the '454 patent. Under Natera's interpretation of the claim, Forshew teaches detection of an SNV because "once you have performed Step 1A and Step 1B and the first part of Step 1C and then you sequence, you will necessarily have identified the SNV if it exists." *See, e.g.*, Metzker 2/11/2025 Dep. Tr. at 29:1-12. Under Natera's apparent interpretation of the claim, Forshew also teaches the 0.015% limitation because Forshew teaches background error rates for SNVs that are below 0.015% (*see, e.g.*, Forshew supplementary materials at Figure S3). *See also, e.g.*, Metzker 2/11/2025 Dep. Tr. at 92:4-93:4, 112:1-114:5, 116:12-24, 125:9-20.

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Claim	**Forshew**
	Forshew discloses "[calling] point mutations in a sample only if nonreference AFs were above the respective substitution-specific background distribution at a high confidence margin (0.9995 or greater), and ranked high in the list of nonreference AFs, in both replicates (Supplementary Methods)." Forshew at 3. As another example, Forshew discloses that "Amplicon libraries were tagged and pooled together for sequencing with libraries prepared from 24 control samples." Forshew at 4. In the context of screening for mutations, Forshew discloses that "Background frequencies of nonreference reads were ~0.1% (median, 0.03%; mean, 0.2%; in keeping with Q30 quality threshold applied), yet varied substantially between loci and base substitutions (Fig. 1C) and showed a clear bias toward purine/pyrimidine conservation (Fig. 1D)." Forshew at 3. In that context, Forshew discloses "we obtained 100% sensitivity, identifying mutations at AFs as low as 0.6%." *Id.* In the context of a tumor-informed assay, Forshew discloses: "When applying TAm-Seq to measure a predefined mutation (as opposed to screening thousands of possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus. False detection is less likely, and criteria for confident mutation detection for a predefined substitution can be less stringent than those described above for de novo mutation identification (Supplementary Methods)." *Id.* Forshew further discloses that "The minimal nonreference AFs that could be detected depend on the read depth and background rates of nonreference reads, which vary per locus and substitution type." *Id.* Forshew further discloses that "[m]inimal detectable frequencies increase when higher confidence margins are used (Supplementary Methods) and had a median value of 0.14% at confidence margin of 0.95 and 0.18% at confidence margin of 0.99 (fig. S3)." *Id.* A POSA reading Forshew would thus understand that much lower SNV frequencies could be detected with lower confidence margins. According to Natera, SNV detection does not require confidence that the SNV is actually present. *See, e.g.*, Metzker 2/11/2025 Dep. Tr. at 79:8-23 (explaining that confidence levels regarding whether ctDNA is actually present are computed downstream of SNV detection), 92:4-93:4, 96:9-19 (explaining that "[a]ccording to the claim language, [detecting an error that matches the SNV at the SNV locus] would be detecting an SNV mutation" even though "[i]n the real world, things don't work that way"). Supplemental Figure S3 shows some minimal detectable frequencies close to or below 0.015%, even with a confidence margin of 90%. Forshew at Figure S3. A POSA reading Forshew would understand that the minimal

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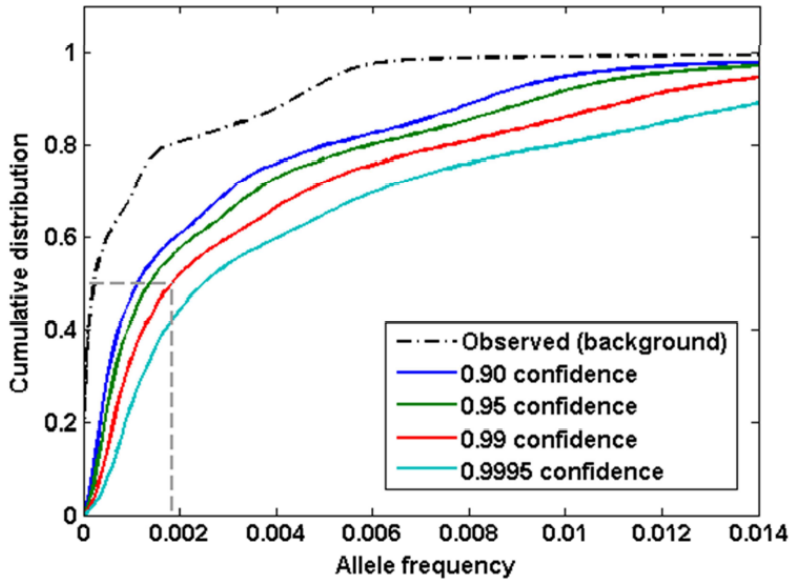
	detectable frequencies would be much lower without a requirement for 90% confidence. *See* Forshew at 3 (explaining the inverse relationship between confidence levels required and minimal detectable SNV frequencies). Additionally, Forshew Figure S3 shows that the observed background error frequency was below 0.015% or below 0.015% for at least a subset of base changes:  Forshew supplementary materials at Figure S3. According to Dr. Metzker's analysis, Forshew's method could, detect mutations that are present at or above the level of background noise in an assay. *See, e.g.*, Metzker 11/14/2023 Dep. Tr. at 98:11-101:13 (using "brown M&Ms" as an analogy for noise and "red M&Ms" as an analogy for ctDNA). Therefore, under Natera's apparent interpretation of this limitation, Forshew discloses a method that is capable of detecting an SNV that is present in less than or equal to 0.015% of the cfDNA because it discloses that background error levels for some substitution types is less than or equal to 0.015%. *See* Forshew Figure S3.

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Claim	**Forshew**
	As another example, Forshew discloses that "For each locus and base, the distribution of non-reference background allele frequencies/reads was modeled as a Normal distribution and as a Poisson distribution, fitting to a Normal distribution and using the mean number of observed nonreference reads in a set of representative barcodes as the parameter for the Poisson distribution." Forshew supplementary materials at 4. As another example, Forshew discloses that "When applying TAm-Seq to measure a pre-defined mutation (as opposed to screening nearly 18,000 possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus. We used confidence margins of ~0.95 using a Normal distribution model for each examined substitution, and requiring a minimum of 10 representative reads, in at least one amplified library per sample. When a mutation was positively detected, its allele frequency was estimated by averaging the allele frequencies in all replicates after subtracting substitution-specific background frequencies." Forshew supplementary materials at 4. As another example, Forshew Figure 1C and Figure 1D (reproduced below) show that the background frequency of nonreference alleles was less than 0.015% or below 0.01% for some SNV loci and types of base substitutions (*see* Forshew at 3 (describing Fig. 1C and Fig. 1D)):

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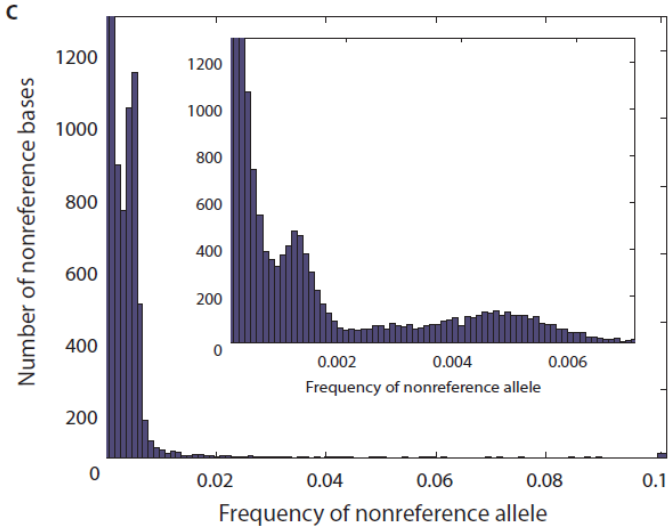
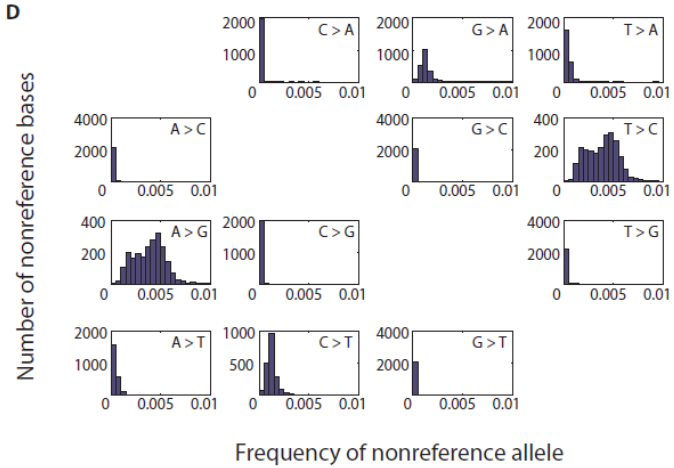
Claim	**Forshew**
	 

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Claim	**Forshew**
	As another example, Forshew discloses:

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	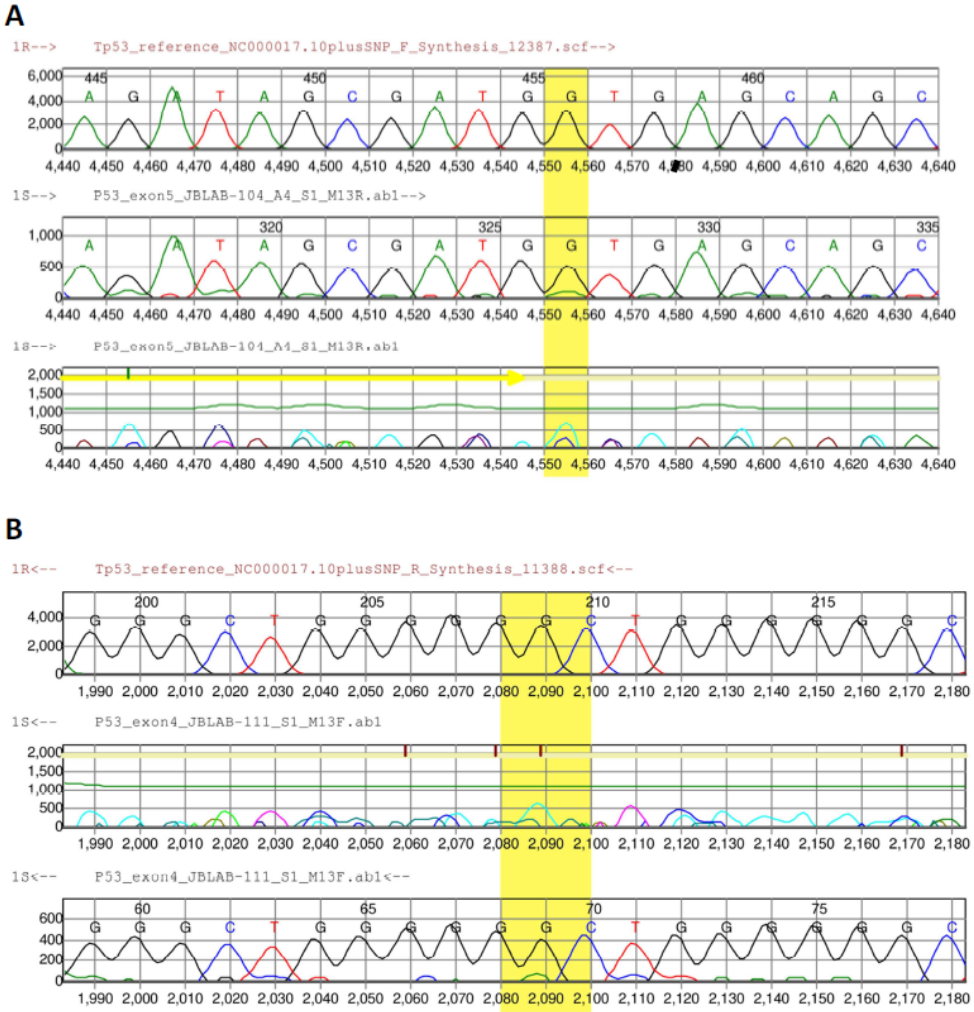

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Claim	**Forshew**
	Forshew supplementary materials at Figure S2. Forshew teaches that identifying patient-specific mutations comprises sequencing matched tumor and normal tissue samples from a patient. *See* Forshew supplementary materials at 19. As to the degree of sensitivity, Forshew specifically teaches the importance of increasing sensitivity and how to improve sensitivity of the TAm-Seq assay, including by increasing sequencing depth and fidelity. Forshew at 10. Further evidence of motivation and reasonable expectation of success is found in NEOGEN00275278–NEOGEN00275334 and NEOGEN00275270–NEOGEN00275277. As an example, NEOGEN00275278 describes experiments performed by Forshew around March 2011 demonstrating successful detection of TP53 mutations in cell-free DNA. For example, NEOGEN00275278 states: "Last week we successfully identified the same mutation by sequencing cell free DNA from the blood directly. To do this we extracted the DNA from blood plasma at two different treatment time points. We then pre amplified the DNA using primers to a number of genes including TP53. We then put this preamplified DNA on a Fluidigm Access Array before sequencing with an Illumina sequencer. We were able to sequence all of TP53 and PTEN and a number of other regions. Through this we identified the same mutation. In the first time point we found that 17% of this DNA was mutated (336/2014 reads). At the second time point this had dropped to 1% (21/2614 reads)." As another example, Forshew discloses: "When applying TAm-Seq to measure a predefined mutation (as opposed to screening thousands of possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus." Forshew at 3. Therefore, Forshew explains, "[f]alse detection is less likely, and criteria for confident mutation detection for a predefined substitution can be less stringent than those described above for de novo mutation identification (Supplementary Methods)." *Id.* As another example, Forshew discloses that "[the] minimal nonreference AFs that could be detected depend on the read depth and background rates of nonreference reads, which vary per locus and substitution type. Minimal detectable frequencies increase when higher confidence margins are used (Supplementary Methods) and had a median value of 0.14% at confidence margin of 0.95 and 0.18% at confidence margin of 0.99 (fig. S3)." Forshew at 3. To the extent Natera contends that the claims do not require a particular confidence threshold for

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	an SNV or a particular clinical sensitivity, a POSA reading Forshew would understand that an SNV that is present at 0.015% could be detected using TAm-Seq with a lower confidence margin. Forshew at 3. As another example, Forshew discloses: "The minimal detectable frequency would also be limited if a minimal number of reads is applied for confident mutation detection; for example, a minimum of 10 reads implies that sequencing depth of 5000 would be required to detect mutations at AF as low as 0.2%. For alleles present at ~10 or fewer copies in the starting template, reproducibility would also be limited by sampling noise, because these alleles may be over- or underrepresented in any particular reaction." Forshew at 3. As another example, Forshew discloses that "[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols." Forshew at 10. NEOGEN00275326 and NEOGEN00275329 provide further evidence of motivation and reasonable expectation of success for increased read depths. *See, e.g.*, NEOGEN00275326 (describing tables plotting the "number of reads for each barcode according to which block of 48 barcodes it belongs to") and NEOGEN00275329 (providing read counts of greater than 50,000 for some loci (e.g., in columns labeled FLD0200, FLD0233, FLD0234, FLD0235, FLD0236, FLD0237, FLD0238, and FLD0239). As Forshew specifically teaches, "[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols." Forshew at 10. *See also* Forshew at 3 ("The minimal nonreference AFs that could be detected depend on the read depth and background rates of nonreference reads… for example, a minimum of 10 reads implies that sequencing depth of 5000 would be required to detect mutations at AF as low as 0.2%."). Gerstung discloses an error-correction method for accurate detection of true SNVs present at less than or equal to 0.015% of the cell-free DNA. *See, e.g.*, Gerstung at Abstract, 1--2, 7, Fig. 1, Table 1, Fig. 2. As an example, Gerstung discloses: "The control sample allows for estimating the local error rate, which increases the power for calling true variants at a given false-positive rate. Unlike true variants, sequencing errors depend on the directionality of sequencing and tend to occur more often on one DNA strand than the other,

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	which can be used to further increase the specificity of variant calling. Batch-library preparation and sequencing in the same run ensure identical noise characteristics of test and control, an important prerequisite for reliable variant detection." Gerstung at 2. As another example, Gerstung discloses: "We show in a rigorous experimental assessment that our approach is capable of detecting variants with frequencies as low as 1/10,000 alleles." Gerstung at Abstract. As another example, Gerstung discloses: "For SNV frequencies larger than or equal to 10^{-4}, the measured nucleotide frequencies accurately agree with the true values, whereas SNVs with frequencies below 10^{-4} are additively biased by sequencing errors that occurred at a median rate of 2.2×10^{-5} (Fig. 2a)." Gerstung at 2, Fig. 2a. As another example, Gerstung discloses that its method, called deepSNV, "calls variants with frequencies higher than 10^{-4} with high sensitivity and specific (Fig. 2c).") Gerstung at 2. As another example, Gerstung discloses that "[a]lthough the power of deepSNV is comparable to that of vipR for variant frequencies of 0.1 and 0.01, its performance is considerably better for variant frequencies of .001 and 0.0001 (Supplementary Fig. S4)". Gerstung at 2. As another example, Gerstung discloses that "[f]or low frequencies of 10^{-3}, deepSNV achieves a power of 86%, compared with the second-best method with 53%." Gerstung at 2. As another example, Gerstung teaches that increasing read depth predictably increases the power to detect an SNV. For example, Gerstung discloses that "[r]oughly, the required coverage for calling a variant needs to be at least ten times higher than its inverse frequency." Gerstung at 2, Gerstung Supplementary Materials at Supplementary Fig. S5C. As another example, Gerstung supplementary figure S5B shows that increasing read depth from 10,000 to 100,000 improves the significant fraction of variants detected at frequencies of 1e-04 (0.0001 or 1 in 10,000).

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	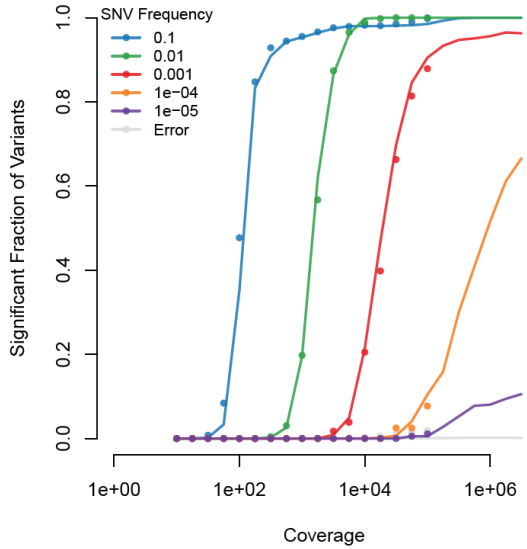 Gerstung Supplementary Materials at Supplementary Figure S5B. As another example, Gerstung discloses that "[t]he current limit of detection is around 1/10,000 alleles, but it may be further improved by increased coverage and higher sequencing fidelity with improved biochemistry or barcoded reads." Gerstung at 4. As another example, Gerstung discloses that "[for] a mixed sample, the distribution of reads with SNVs (grey arrows with coloured dots) resembles the population structure, but sequencing errors (black dots) confound calling of SNVs. A homogeneous control sample allows for precise estimation of the local error rate, which is often biased to one strand, and enables accurate SNV calling against the background noise frequency." Gerstung at 3 (Figure 1 legend). *See also* Gerstung Figure 1(a), reproduced below (showing how precise estimation of local error rate enables accurate SNV calling).

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	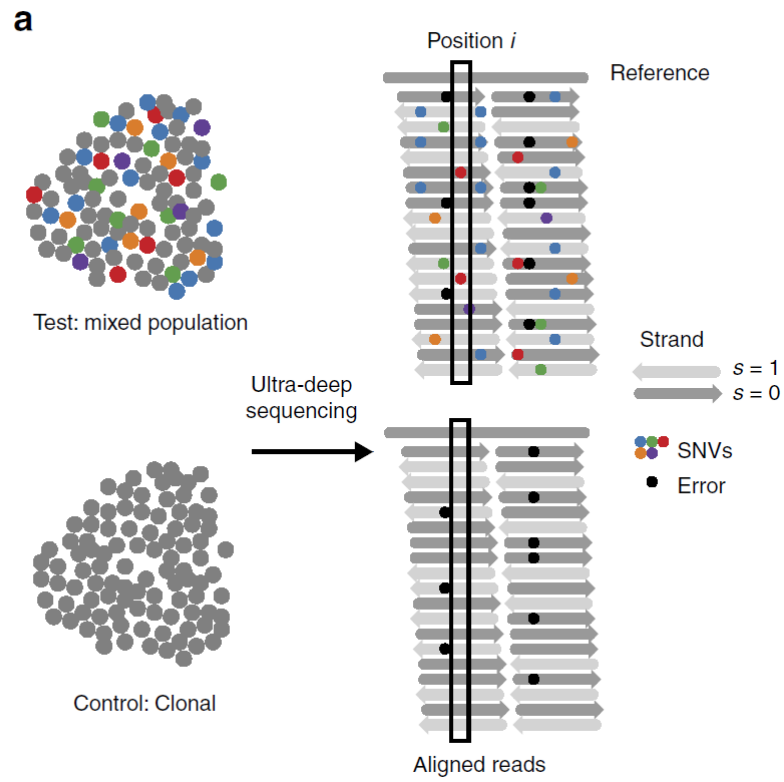 Kukita discloses detecting an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus. *See, e.g.*, Kukita at 1-3, For example, Kukita teaches "The detection of rare mutants using next generation sequencing has considerable potential for diagnostic applications. Detecting circulating tumor DNA is the foremost application of this approach. The major obstacle to its use is the high read error rate of next-generation sequencers. Rather than increasing the accuracy of final sequences, we detected rare mutations using a semiconductor sequencer and a set

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	of anomaly detection criteria based on a statistical model of the read error rate at each error position. Statistical models were deduced from sequence data from normal samples. We detected epidermal growth factor receptor (EGFR) mutations in the plasma DNA of lung cancer patients. Single-pass deep sequencing (>100,000 reads) was able to detect one activating mutant allele in 10,000 normal alleles. We confirmed the method using 22 prospective and 155 retrospective samples, mostly consisting of DNA purified from plasma. A temporal analysis suggested potential applications for disease management and for therapeutic decision making to select epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI)." Kukita at 1. As another example, Kukita teaches "With an increasing read depth and decreasing RER, the detection limit decreases. In a previous study by our group [7], the detection limit for rare mutant alleles when using BEAMing [4] was 1 in 10,000 (0.01%). Because a plasma DNA assay sample contains approximately 5,000 molecules, this detection limit is reasonable. This goal can be achieved with 100,000 reads when the RER is below 0.01%." Kukita at 2. As another example, Kukita teaches Figure 1a:

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	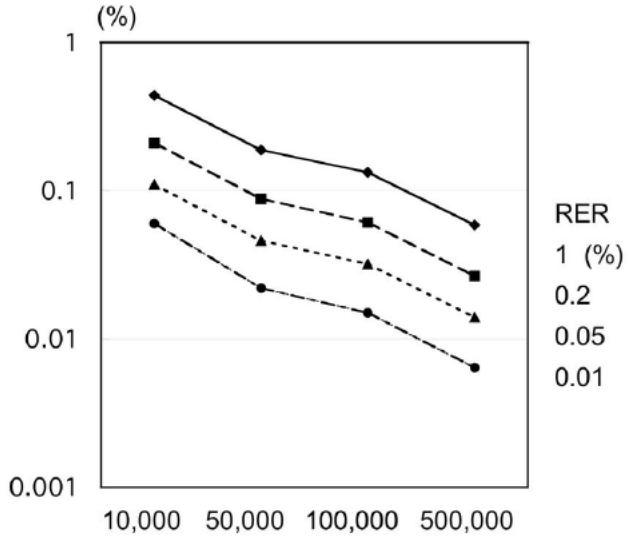 Kukita at 3. Kukita teaches using an "Ion Torrent PGM sequencer for this work" and that "Single-pass sequencing was performed, and the number of reads ranged from 44,400 to 373,000, averaging 162,000." Kukita at 2. As another example, Kukita teaches that "In 410 out of the possible 506 types of substitution (81.0%), the RER was lower than 0.01%." Kukita at 2. As another example, Kukita discloses: "Deep sequencing of a PCR-amplified fragment containing a mutation site can be conducted to detect and quantitate mutated alleles among the vast amounts of normal alleles derived from host tissues. The major problem associated with this approach is the frequency of errors introduced during sequencing and PCR amplification. The key issue here is the setting and accurate evaluation of detection limits. When the frequency of a base change at a target locus is higher than a predetermined read error rate (RER), we

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may judge the change to be due to the presence of a mutant sequence. That is, anomalies that fall significantly outside of the RER distribution are regarded as mutations." Kukita at 2.

As another example, Kukita discloses supplementary Table S1:

RER	λ (number of read errors)	number of reads	threshold (P<0.01)	detection limit (%)(P<0.01)	threshold (P<2x10−5)	detection limit (%)(P<2x10−5)
1%	10	1000	18	0.8	25	1.5
	100	10000	124	0.24	144	0.44
	500	50000	553	0.106	594	0.188
	1000	100000	1075	0.075	1133	0.133
	5000	500000	5165	0.033	5293	0.0586
0.20%	2	1000	6	0.4	10	0.8
	20	10000	31	0.11	41	0.21
	100	50000	124	0.048	144	0.088
	200	100000	234	0.034	261	0.061
	1000	500000	1074	0.0148	1133	0.0266
0.05%	5	10000	11	0.06	16	0.11
	25	50000	37	0.024	48	0.046
	50	100000	67	0.017	82	0.032
	250	500000	288	0.0076	320	0.014
0.01%	1	10000	4	0.03	7	0.06
	5	50000	11	0.012	16	0.022
	10	100000	18	0.008	25	0.015
	50	500000	67	0.0034	82	0.0064
0.005%	1	10000	4	0.03	7	0.06
	3	50000	8	0.01	12	0.018
	5	100000	11	0.006	16	0.011
	25	500000	37	0.0024	48	0.0046

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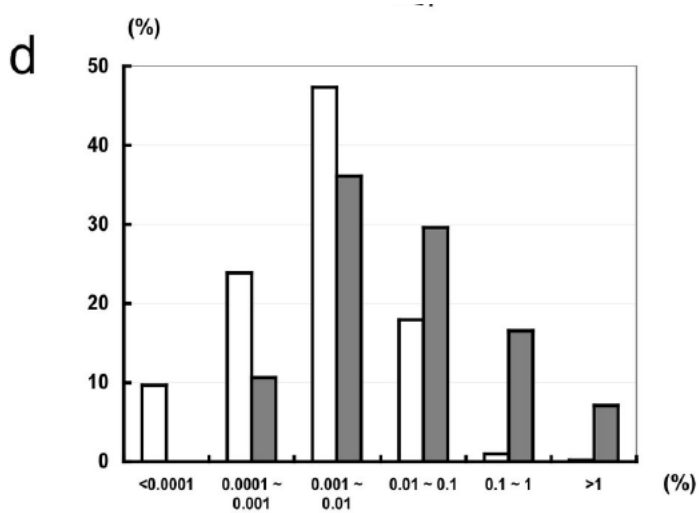
	As another example, Kukita discloses Figure 1(d) (showing distribution of the RER (%. White column, substitution error):  *See, e.g.,* Patel at 1, 3-4, Fig. 4B, 36, ("Mutant DNA could be accurately and reproducibly measured in a linear manner over approximately 8 orders of magnitude and down to levels as low as 1 in 10,000 molecules."), Fig. 6 (showing accurate and reproducible detection of SNVs down to levels as low as 1 in 10,000 molecules) (Fig. 1 illustrating an example of error-suppressed deep sequencing involving multiplex PCR of target loci). Patel further states that it describes "[m]ethods and compositions… that provide a multiplex assay to detect minute amounts of ctDNA." Patel at 1. As an example, to achieve error-suppressed sequencing, Patel discloses using primers comprising "a barcode and/or a molecular lineage tag (MLT)." *See, e.g.*, Patel at 2. Patel discloses that MLT sequences can be used to "trace the lineage of molecules from initial copying through amplification, processing, and sequencing" and "can be used to distinguish sequences that arise from polymerase misincorporations or sequencer errors from

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	sequences that are derived from true mutant template molecules." Patel at 13. *See also* Patel at 18-19 (describing the benefits and use of MLTs to distinguish variant sequences arising from true mutant template molecules versus those arising from misincorporation errors). As another example, Patel discloses overlapping paired-end reads covering a mutation hotspot from the forward and reverse strands of each clone "yields highly accurate base-calls, permitting detection and quantification of rare mutations with greater sensitivity." Patel at 3-4, Fig. 4B. As another example, Patel discloses that sequencing reads from opposite strands "provide sequence redundancy via overlap in the mutation-prone region" and that "[this] clonal redundancy allowed sequences resulting from sequencer errors to be identified and discarded, permitting greater sensitivity for detection of rare sequence variants." Patel at 7, Fig. 14. Patel at Fig. 15 further explains how analysis of variant sequences can be performed. *See also* Patel at 10-11 (explaining the benefits of clonal overlapping paired-end sequencing to greatly reduce the background of sequencer-generated errors and allow rare mutant molecules to be detected with greater sensitivity). A POSA would understand that Patel could be combined with the tumor-specific known SNVs described in Forshew to obtain overlapping reads for the SNV of interest, similarly to the method for obtaining overlapping reads for a mutation region. As another example, Patel discloses that: "Figs. 6A-6C indicates the performance of error-suppressed deep sequencing. Measurements of DNA extracted from mutant and wild-type cancer cell lines mixed in various ratios ranging from 1:10,000 to 10,000:1 show a high degree of accuracy and reproducibility." Patel at 4, Figs. 6A-6C. As another example, Patel discloses how sensitivity of sequencing is typically limited by the inherent error rate of the sequencer "since incorrectly read bases might be mistaken for true mutant DNA copies." Patel at 17-18. Patel further discloses how the error-suppressed PCR methods address sequencing errors, including by using "clonal overlapping paired-end sequencing to achieve sequence redundancy in mutation-prone regions, thereby allowing base calls to be made with much greater confidence." *Id.* As another example, Patel explains that its methods for error-suppressed sequencing allow for "distinguishing nucleotide misincorporation errors that can be introduced during DNA copying, amplification, or processing." Patel at 18.

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	As another example, Patel discloses that when ultra-deep sequencing is performed, "[in] principle, because large numbers of sequences can be obtained for each target site and for each sample, rare variants can be detected and measured." Patel at 27. However, Patel discloses that "the error rate of the sequencer can limit the sensitivity of detection because such errors might be mistaken as true variants." *Id.* Patel discloses a solution for improved sensitivity of detection for rare SNVs because "errors introduced into the DNA during amplification or processing can be distinguished from true template-derived mutant sequences by analyzing the distribution of molecular lineage tags (MLTs) associated with variant sequences." *Id.* As another example, Patel discloses that its method of error-suppressed sequencing was able to reduce background levels of spurious mutation counts to levels below 0.015%, including (for some SNVs), several-fold or even ten-fold below 0.015% (0.00015). *See* Patel at 36, Table 3:

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Table 3: Background level of spurious mutation counts obtained from known wild-type samples.

Mutation Type	Fraction of mutant : wild-type counts (from 3 replicate PCRs)
BRAF Val600Glu	0.00013
EGFR Leu858Arg	0.000047
KRAS Gly12Ser	0.00029
KRAS Gly12Val	0.00018
KRAS Gly12Arg	0.000057
KRAS Gly12Asp	0.00015
KRAS Gly12Ala	0.000014
KRAS Gly12Cys	0.00025
KRAS Gly13Ser	0.00015
KRAS Gly13Val	0.00029
KRAS Gly13Arg	0.000050
KRAS Gly13Asp	0.000044
KRAS Gly13Ala	0.000058
KRAS Gly13Cys	0.00049
Median Value	**0.00014**
Interquartile range	**0.000052 to 0.00023**

As another example, Patel discloses that its method was able to detect SNVs down to levels as low as 1 in 10,000 molecules (0.01% VAF), while also being able to "simultaneously quantify more than one mutation from a given sample" using targeted multiplex amplification and sequencing. *See* Patel at 36.

</td></tr>
<tr><td>15. The method of claim 14, wherein the sequencing has a depth of read of at least</td><td>Forshew discloses the sequencing has a depth of read of at least 50,000 per target locus.

See also claim 1[d] and 14[b], [c], [d] above.</td></tr>
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50,000 per target locus.	
22. The method of claim 14, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation.	Forshew discloses that the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation. *See* claim 8 above.
24. The method of claim 14, wherein the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample.	Forshew discloses that the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample. *See* claim 10 above.
26. The method of claim 14, wherein the method further comprises detecting recurrence and/or	Forshew discloses that the method further comprises detecting recurrence and/or metastases of the cancer from the SNV mutations detected in the cell-free DNA. *See* claim 12 above.

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metastases of the cancer from the SNV mutations detected in the cell-free DNA.	
28. The method of claim 14, wherein the method is capable of detecting an SNV mutation that is present in 0.005% to 0.015% of the cell-free DNA comprising the SNV locus.	Forshew discloses the method that is capable of detecting an SNV mutation that is present in 0.005% to 0.015% of the cell-free DNA comprising the SNV locus. *See* claims 1[d] and 14[d] above.

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