EXHIBIT 1-B — FINAL INVALIDITY CLAIM CHART FOR U.S. PATENT NO. 11,319,596

References: Forshew T. et al., *Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA,* 4 Sci. Transl. Med. 136 (May 30, 2012) ("Forshew")

To the extent Natera asserts that Forshew does not disclose or teach one or more limitations of asserted claims 1, 11, 12, or 18, 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.* 3 Nat. Commc'ns 811 (May 1, 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,* 8 PLOS ONE11, e81468 (Nov. 13, 2013) ("Kukita")

Reference: Bashashati A. et al., *Distinct evolutionary trajectories of primarily high-grade serous ovarian cancers revealed through spatial mutational profiling*, 231 J. Pathol. 21–34 (June 7, 2013) (including its supplementary appendix and supplementary spreadsheet) ("Bashashati")

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").

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A POSA would also be motivated to combine Forshew with Gerstung or Kukita 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 Title ("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 Abstract ("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"). A POSA would therefore be motivated to turn to references such as Gerstung or Kukita 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.

A POSA would have a reasonable expectation of success in combining Forshew with Gerstung or Kukita 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 and Kukita 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 "above the respective substitution-specific background distribution at a high confidence margin" (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, 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

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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 sequencing depth" by "simply sequenc[ing] more." *See, e.g.,* Illumina, *Estimating Sequencing Coverage,* Technical Note: Sequencing, (2014) ("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%, including at high read depth. *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 at a high confidence margin" (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-Sesq 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 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

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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 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. 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 1) 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 "[t]he major obstacle [of] high read error rate of next-generation sequencers" and achieve "[t]he detection of rare mutants using next generation sequencing." Kukita at Abstract.

A POSA would be motivated to combine Forshew with Gerstung or Kukita and 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* 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).

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Like Forshew, Gerstung discloses that "additional sensitivity for calling low-frequency variants can be gained by increas[ing] 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.

A POSA would be motivated to modify the method of Forshew with Bashashati to use whole-exome sequencing (WES) rather than whole-genome sequencing (WGS) to identify the tumor-specific mutations in the tumor biopsy sample because WES is cheaper than WGS. And Forshew teaches that performing whole-genome or whole-exome sequencing were well known alternatives in the art. *See, e.g.*, Forshew at 9 ("Advances in massively parallel sequencing make new approaches possible. These have largely focused on large-scale analyses, including whole-genome or whole-exome sequencing.").

A POSA would also have a reasonable expectation of success in modifying the method of Forshew with Bashashati to use whole-exome sequencing (WES) rather than whole-genome sequencing (WGS) because Forshew teaches that the 10 SNV mutations identified in the breast cancer tumor sample for tracking in cfDNA were mutations in gene exons. *See,* Forshew at Table S7; *see also* Forshew at Tables S3 and S6 (disclosing SNV mutations identified in exonic regions for ovarian cancer patients). Therefore, a POSA would understand that the cheaper method of WES could be used to identify the tumor-specific mutations.

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Claim	**Forshew**
1[pre]. A method for preparing biological samples useful for	To the extent the preamble is construed to be limiting, Forshew discloses a method for preparing biological samples useful for monitoring the progression of cancer in a subject.

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Claim	**Forshew**
monitoring the progression of cancer in a subject, the method comprising:	*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 collected over the 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 sequencing on a tumor biopsy sample of the subject to identify a plurality of tumor-specific mutations, wherein the tumor-specific mutations comprise one or more single nucleotide variant (SNV) mutations;	Forshew discloses performing sequencing on a tumor biopsy sample of the subject to identify a plurality of tumor-specific mutations, wherein the tumor-specific mutations comprise one or more single nucleotide variant (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

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Claim	**Forshew**
	"[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. 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.
1(b). evaluating results of the sequencing on the tumor biopsy sample to determine a plurality of target loci specific to the subject, wherein each target locus spans a tumor-specific mutation of the identified plurality of tumor-specific mutations; and	Forshew discloses evaluating results of the sequencing on the tumor biopsy sample to determine a plurality of target loci specific to the subject, wherein each target locus spans a tumor- specific mutation of the identified plurality of tumor-specific mutations. *See, e.g.,* Forshew at 3, 9, 10, Table S3, Table S7. For example, Forshew teaches "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. 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.
1(c). assaying cell-free DNA isolated from a plurality of biological samples obtained from the subject at different time points, wherein the assaying comprises:	Forshew discloses assaying cell-free DNA isolated from a plurality of biological samples obtained from the subject at different time points. *See, e.g.,* Forshew at 2, 4, 7, 8, 9, Figs. 4B-4D, and Supplementary Materials at 19 (Table S7). For example, Forshew discloses: "We studied serial plasma samples collected during follow-up and treatment of two patients with relapsed HGSOC, collected during 104 and 273 days of follow-up and treatment, respectively." Forshew at 8. 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 . . . . Serial plasma samples were collected over the 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. (Fig. 4D)." Forshew at 9, Fig. 4D. As another example, Forshew discloses: "Retrospective analysis by TAm-Seq of FFPE from the primary tumors collected at initial surgery, and three plasma samples collected serially at the time of relapse (5 years and 5 months, 5 years and 7 months, and 6 years after initial surgery), showed that the patient' s plasma at relapse contained the TP53 (p.R273H) mutation identified in the ovarian primary tumor." Forshew at 9. As another example, Forshew discloses that "[s]ensitive methods for detecting cancer mutations in plasma" may be useful for "monitoring tumor dynamics over time." Forshew at 1. Forshew further

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Claim	**Forshew**
	teaches that this "sequencing approach allowed us to monitor changes in tumor burden by sampling only patient plasma over time." Forshew at 2. As another example, Forshew discloses: "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." Forshew at Table S7. As another example, Forshew discloses: "DNA extracted from 62 additional plasma samples collected at different time points from 37 patients with advanced HGSOC was amplified in duplicate (table S6), using DNA present in ~0.15 ml of plasma per reaction (range, 0.06 to 0.2 ml)." Forshew at 4. As another example, Forshew Fig. 4B, 4C, and 4D all show results involving assaying cell-free DNA obtained at multiple time points to monitor the frequency of mutant DNA in plasma over time. *See* Forshew at 7 (Fig. 4B, Fig. 4C, Fig. 4D, and accompanying legend). Kukita teaches assaying cell-free DNA isolated from a plurality of biological samples obtained from the subject at different time points. *See, e.g.*, Kukita at 5-8. For example, Kukita teaches "We then analyzed a total of 155 samples: 144 samples from plasma, eight from cerebrospinal fluid, and one each from urine, pleural effusion, and bronchial alveolar lavage. As for plasma samples, two or more samples were obtained from 32 patients at different time points of the disease courses." Kukita at 5. As another example, Kukita teaches "A considerable number of samples were collected from the same patient at different time points in the disease course. Temporal changes of EGFR mutation levels in plasma DNA from patients with three or more samples are schematically shown in Figure 3." *Id.* As another example, Kukita teaches Figure 3:

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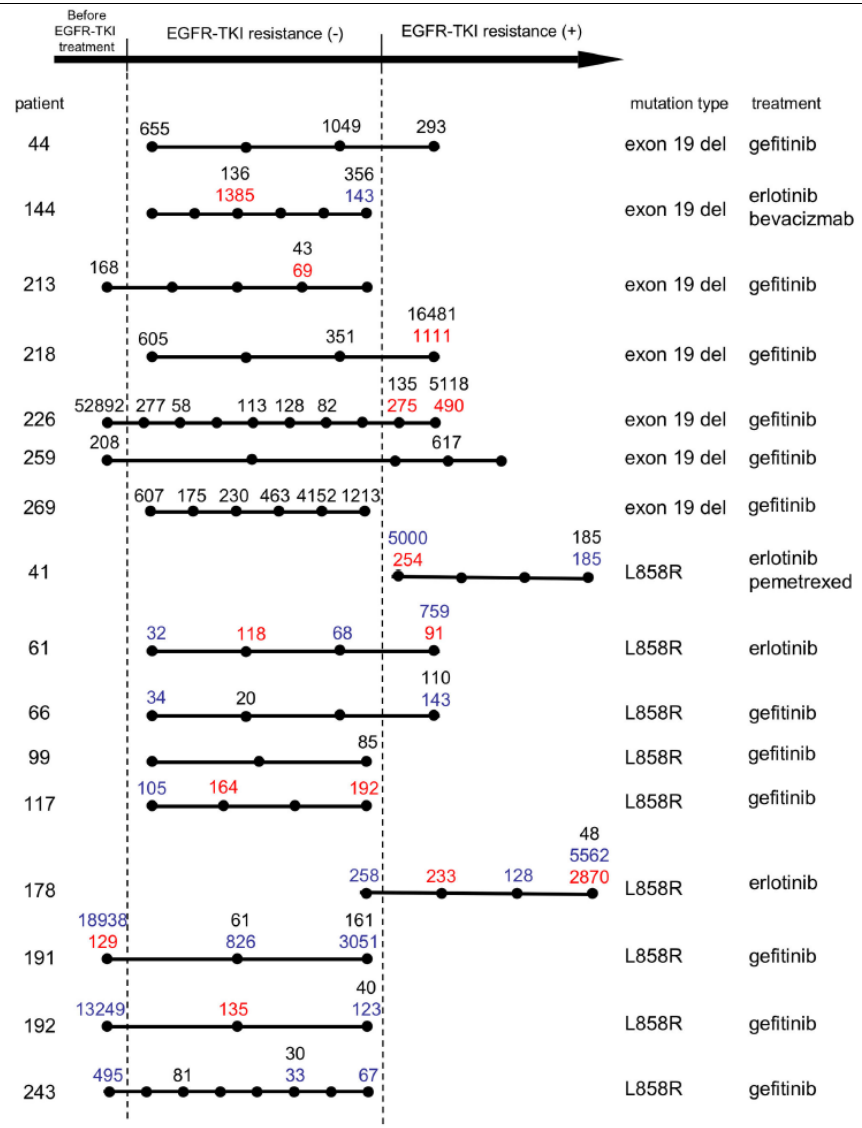

Figure 3. Temporal changes of *EGFR* mutations in plasma DNA from patients with three or more samples. Each dot represents a time point of sampling. The diagram is not precise representation of time scale, and only the order of dots is valid information. Figures represent *EGFR* mutations in 10,000 sequence reads: black, exon 19 deletion; blue, L858R; red, T790M. Only figures exceeding the thresholds are shown. "Mutation type" indicates that in the biopsy samples.

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Claim	**Forshew**
	Kukita at 6. As another example, Kukita teaches Figure S2:

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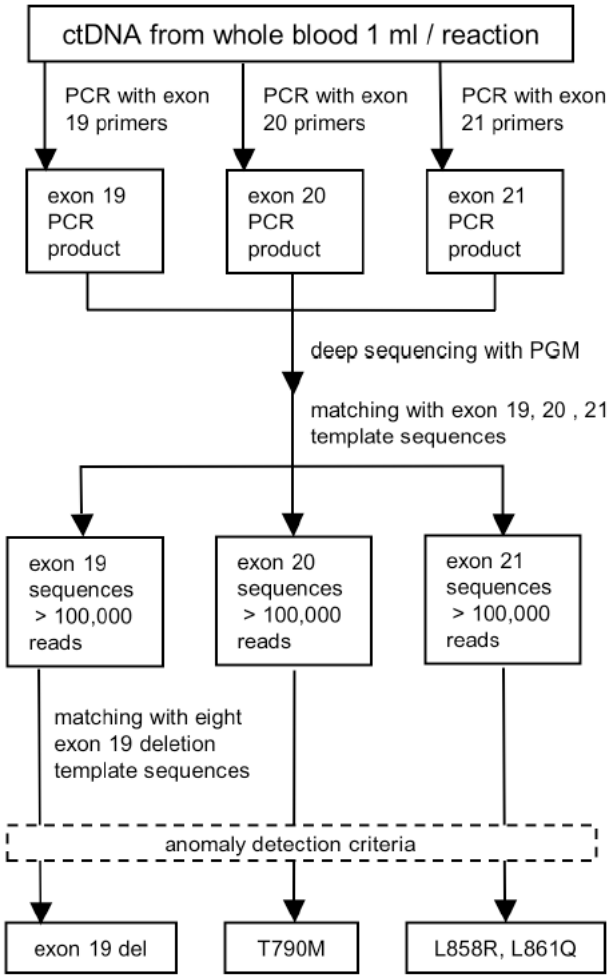

Figure S2. Outline of the method.

Kukita supporting information at Figure S2.

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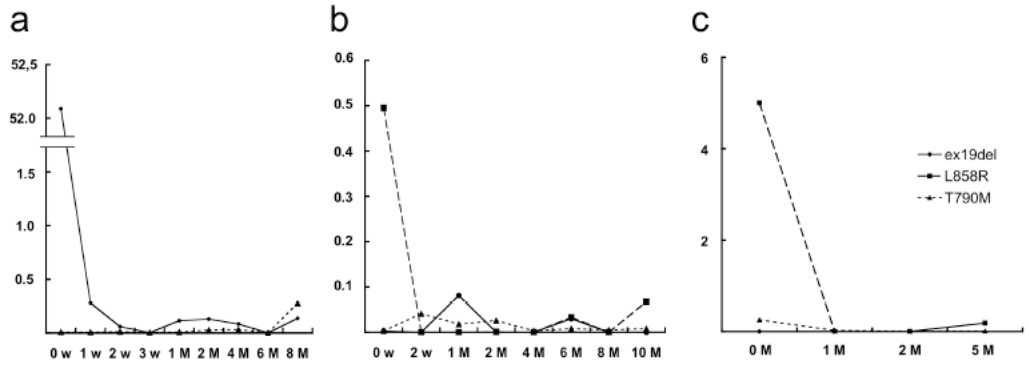
Claim	**Forshew**
	As another example, Kukita teaches Figure S3:  Figure S3. Dynamics of mutant alleles in plasma cell-free DNA during EGFR-TKI treatment. a, Patient 226, b, Patient 243, c, Patient 41. Horizontal axis, time point after the initiation of gefitinib treatment (a, b) or erlotinib-pemetrexed treatment (c); vertical axis, mutant allele frequency (%). Kukita supporting information at Figure S3.
1(c)[1]. performing targeted multiplex PCR amplification to amplify the plurality of target loci together in the same reaction volume from the isolated cell-free DNA	Under Natera's apparent interpretation of "the isolated cell-free DNA", Forshew discloses performing targeted multiplex PCR amplification to amplify the plurality of target loci together in the same reaction volume from the isolated cell-free DNA using primers specific to the plurality of target loci for the individual subject. *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

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using primers specific to the plurality of target loci for the individual subject; and	"[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 at 4 ("We applied TAm--Seq to directly identify mutations in plasma of cancer patients."); 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 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 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).

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1(c)[2]. performing high-throughput sequencing of the amplified DNA comprising the plurality of target loci to obtain sequence reads,	Under Natera's apparent interpretation of this term, Forshew discloses performing high-throughput sequencing of the amplified DNA comprising the plurality of target loci to obtain 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."). 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. 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

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	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(c)[3]. wherein an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA having the SNV locus is detected from the sequence reads	In view of Forshew alone or in combination and the knowledge of a POSA, it would have been obvious to detect an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus. A POSA reading Forshew would understand that Forshew's method enables a POSA to detect an SNV that is present in less than or equal to 0.015% of the cfDNA. Obtaining greater sensitivity by increasing sequencing depth was also well-known in the art and a POSA would have had a reasonable expectation of achieving a sensitivity of 0.015% because this had already been accomplished before the '596 patent's priority date. *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, the asserted claims of the '596 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* 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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	Forshew discloses "call[ing] 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 with 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, 96:9-96:19 (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").

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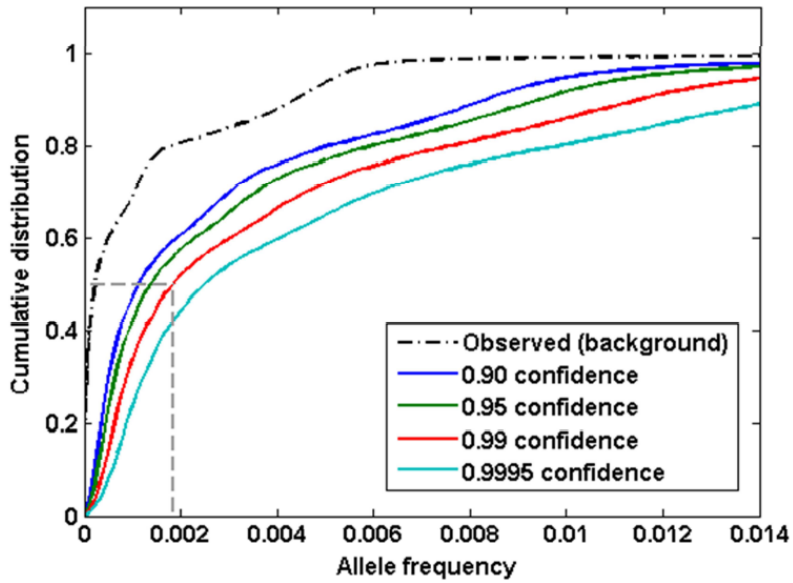
	Supplemental Figure S3 shows some minimal detectable frequencies close to or below 0.015%, even with a confidence margin of 90%. Forshew at Figure S3. A POSA reading Forshew would understand that the minimal detectable frequencies would be much lower without a requirement for 90% confidence. *See* Forshew 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 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 in which "an SNV mutation that is present in less than or equal

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	to 0.015% . . . is detected" because it discloses that background error levels for some substitution types is less than or equal to 0.015%. *See* Forshew Figure S3. 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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	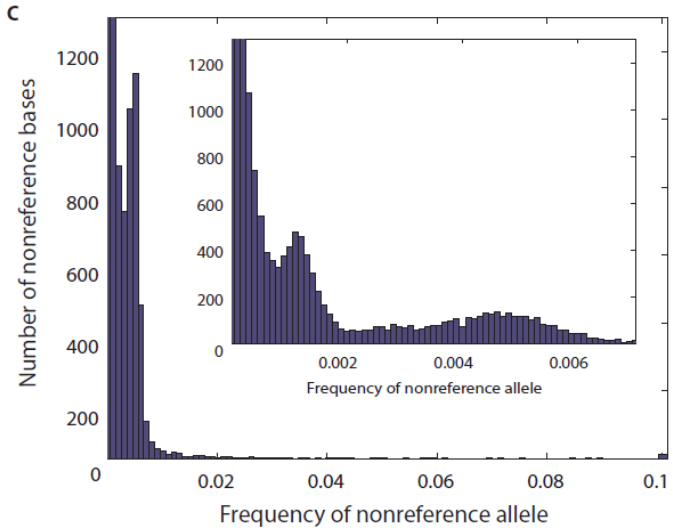 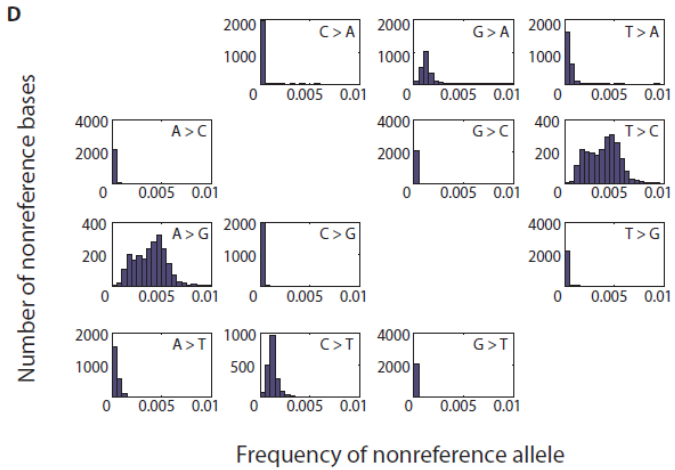

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	As another example, Forshew discloses:

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	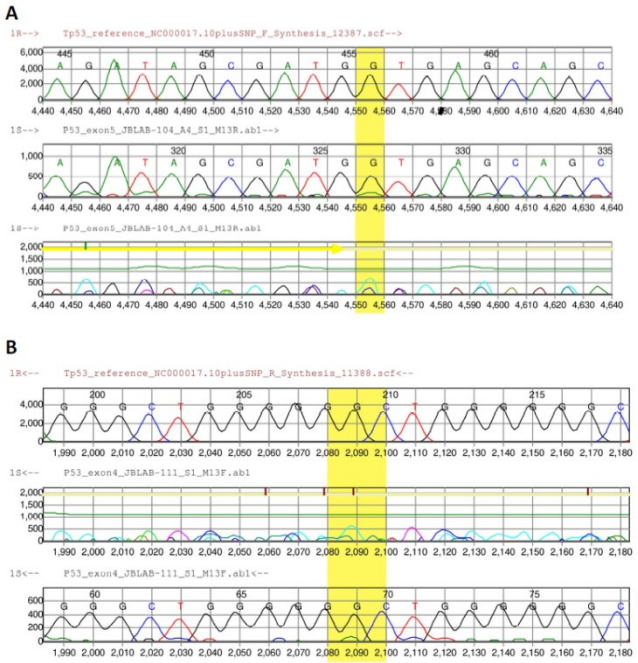 Forshew supplementary materials at Figure S2. Forshew teaches that identifying patient-specific mutations comprises sequencing matched tumor and normal tissue samples from a patient. *See* Forshew supplementary materials at 19. As to the degree of sensitivity, Forshew specifically teaches the importance 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–NEOGEN0027527. As an example, NEOGEN00275278 describes experiments performed by Forshew around March 2011 demonstrating successful detection of TP53 mutations in cell-free DNA. For example,

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	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 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. 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." *Id.*

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	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% VAF. *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, 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 1 (Abstract).

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	As another example, Gerstung discloses sequencing samples to "69,203 to 117,180× coverage." Gerstung at 2. 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 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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	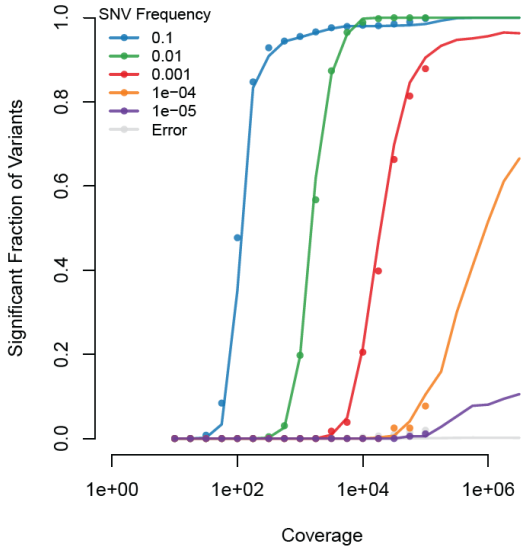 Gerstung Supplementary Materials at Supplementary Figure S5B. As another example, Gerstung discloses that "[t]he current limit of detection is around 1/10,000 alleles, but it may be further improved by increased coverage and higher sequencing fidelity with improved biochemistry or barcoded reads." Gerstung 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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	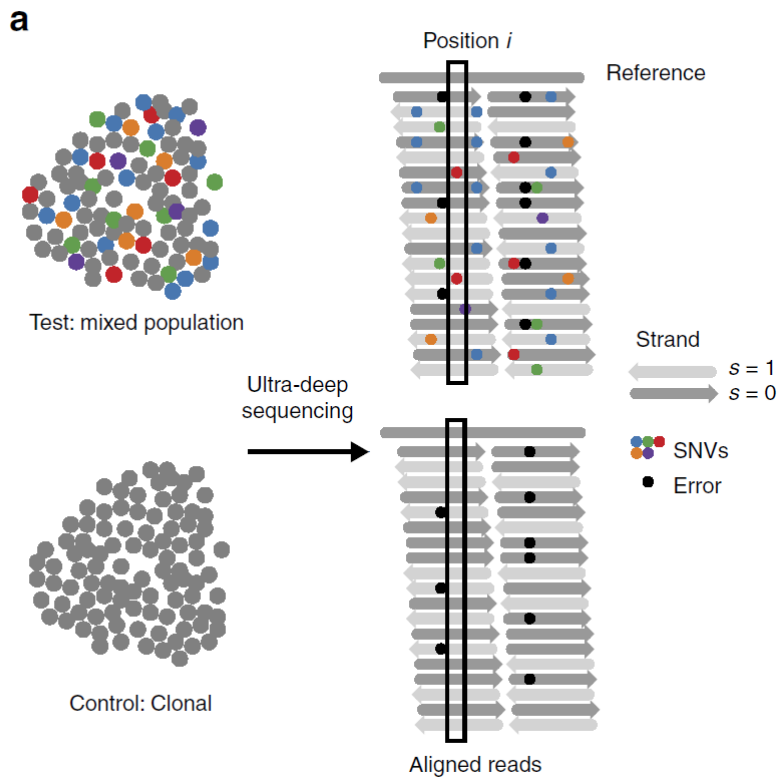 Kukita discloses an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA having the SNV locus is detected from the sequence reads. *See, e.g.*, Kukita at 1, 2, 3, For example, Kukita teaches "The detection of rare mutants using next generation sequencing has considerable 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

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	semiconductor sequencer and a set 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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	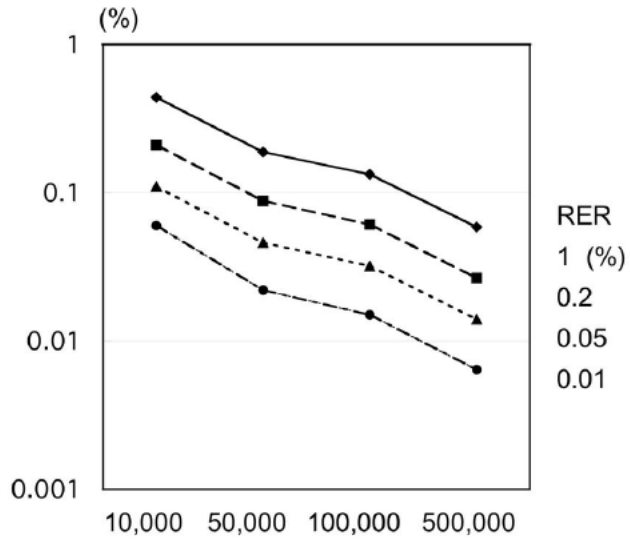 Kukita at 3. Kukita teaches using an "Ion Torrent PGM sequencer for this work" and that "Single-pass sequencing was performed, and the number of reads ranged from 44,400 to 373,000, averaging 162,000." Kukita at 2. As another example, Kukita teaches that "In 410 out of the possible 506 types of substitution (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

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	locus is higher than a predetermined read error rate (RER), we 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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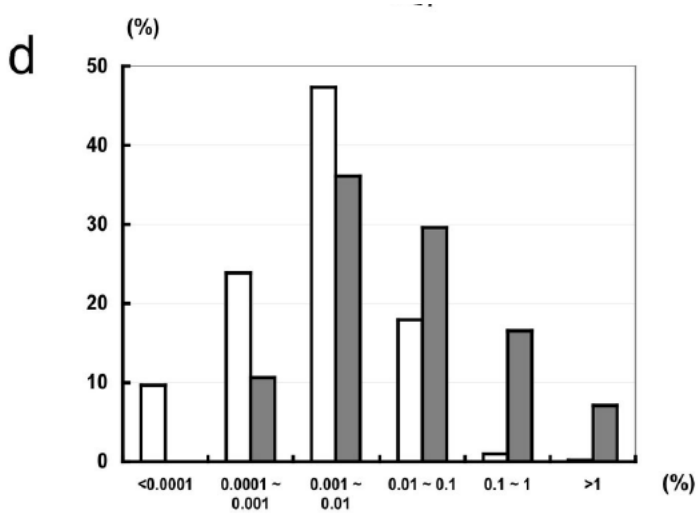
	As another example, Kukita discloses Figure 1(d) (showing distribution of the RER (%). White column, substitution error):  Kukita at 3.
11. The method of claim 1, wherein amplifying comprises targeted multiplex PCR amplification of 10 to 50 of the target loci.	Forshew discloses that the amplifying comprises targeted multiplex PCR amplification of 10 to 50 of the target loci. *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

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	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). 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 10 to 500 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

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	10 to 50 different loci from cfDNA in a single reaction, and 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.
12. The method of claim 1, wherein the method further comprises detecting recurrence and/or metastases of the cancer from the tumor- specific mutations detected in the cell-free DNA.	Forshew discloses wherein the method further comprises detecting recurrence and/or metastases of the cancer from the tumor- specific 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%.").
18. The method of claim 1, wherein step (a) comprises performing whole exome sequencing on a tumor biopsy sample of the subject to identify the plurality of tumor-specific mutations.	In view of Forshew alone or in view of the knowledge of a POSA, it would have been obvious to perform whole exome sequencing on a tumor sample of the subject to identify the plurality of tumor-specific SNV mutations. Forshew discloses performing whole genome sequencing on a tumor sample of a subject to identify the plurality of tumor-specific mutations. *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").

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	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. 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. A POSA would be motivated to modify the method of Forshew to use whole-exome sequencing (WES) rather than whole-genome sequencing (WGS) to identify the tumor-specific mutations in the tumor biopsy sample because WES is cheaper than WGS. A POSA would also have a reasonable expectation of success in modifying the method of Forshew to use whole-exome sequencing (WES) rather than whole-genome sequencing (WGS) because Forshew teaches that the 10 SNV mutations identified in the breast cancer tumor sample for tracking in cfDNA were mutations in gene exons. *See,* Forshew at Table S7; *see also* Forshew at Tables S3 and S6 (disclosing SNV mutations identified in exonic regions for ovarian cancer patients). Therefore, a POSA would understand that the cheaper method of WES could be used to identify the tumor-specific mutations.

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	Bashashati discloses performing whole exome sequencing on a tumor biopsy sample of the subject to identify the plurality of tumor-specific mutations. *See, e.g.,* Bashashati supplementary app'x at 5–6, 6; Bashashati at 22. For example, Bashashati discloses that "Genomic DNA was extracted from frozen samples using the Gentra Puregene kit (Qiagen) and quantified using a Qubit fluorometer (Invitrogen). Exome capture was achieved through solution hybrid selection with the Human All Exon kit SureSelect Target Enrichment System (Agilent) version 1 for Illumina Genome Analyzer paired-end sequencing (Gnirke et al., 2009) . . . Using the resulting tumour and normal pair of BAM files, somatic SNVs were called by JointSNVMix algorithm (Roth et al., 2012), sorted by marginal posterior probability and further run through the mutationSeq algorithm (Ding et al., 2012) to find high confidence candidate SNVs." Bashashati supplementary app'x at 6. As another example, Bashashati discloses a method in which "the exomes of 25 samples (19 tumour, six normal; Table 1) were sequenced to a median exonic coverage of 70.7-fold across different samples (see supplementary material, Table S1 and Figure S1)." Bashashati at 22. As another example, Bashashati discloses a method in which "multiple tissue samples were obtained from primary ovarian tumor and metastatic sites where adequate tumor volume permitted," teaching that "[g]enomic DNA was extracted from frozen samples" and that "[e]xome capture was achieved through solution hybrid selection with the Human All Exon kit SureSelect Target Enrichment System (Agilent) version 1 for Illumina Genome Analyzer paired-end sequencing."). Bashashati supplementary app'x at 5–6. A POSA would understand that this method discloses whole exome sequencing because Bashashati is using the "Human All Exon kit." As another example, Bashashati teaches that "[c]andidate SNVs and indels were subjected to deep sequencing using Illumina and Ion Torrent platforms" in order to validate mutations. Bashashati supplementary app'x at 6.

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