

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

References: Diaz, LA Jr. and A. Bardelli, *Liquid Biopsies: Genotyping Circulating Tumor DNA*, J. Clin. Oncol. 20;32(6):579-86 (Feb. 20, 2014) (“Diaz”).

To the extent Natera asserts that Diaz 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 Forsheiw in combination with the knowledge of a POSA and one or more of the following references:

Reference: Young, AL et. al., *Quantifying ultra-rare pre-leukemic clones via targeted error-corrected sequencing*, Leukemia 29, 1608–1611 (Feb. 20, 2015) (including its Supplementary Tables and Figures and Supplementary Methods, “Young”).

Reference: Kinde I. et al., *Evaluation of DNA from the Papanicolaou Test to Detect Ovarian and Endometrial Cancers*, Sci Transl Med. 9;5(167):167ra4 (Jan. 2013) (including its supplementary tables and Supplementary Materials, “Kinde”).

Reference: Forsheiw, T. et al., *Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA*, 4(136):136RA68 (May 30, 2012) (“Forsheiw”)

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.

A POSA would also have been motivated to combine Diaz with Forsheiw because both references relate to the same field, namely, detection of mutations in ctDNA.

A POSA would also have been motivated to apply the teachings of Forsheiw in the context of Diaz’s teachings. Diaz discloses: “Recently, PCR-based digital approaches have been updated with techniques that use next-generation sequencing (NGS) to identify rare mutant variants in complex mixtures of DNA (Table 1). These techniques have expanded the ability to detect a single point

mutation, and now multiple genes of interest can be investigated in one sample.” Diaz at 580. Diaz expressly cites Forsheiw for this teaching, and Forsheiw provides one such assay. A POSA would have had a reasonable expectation of successfully considering Forsheiw’s teaching in the context of Diaz because Diaz expressly teaches that Forsheiw’s TAm-Seq method of a tumor-informed assay involving multiplex amplification and sequencing of patient-specific target mutations including a target SNV can be used to detect multiple single point mutations in one sample. *Id.* Diaz further teaches that Forsheiw’s TAm-Seq methods can be implemented to detect single point mutations with a sensitivity of 0.01% or lower. *Id.* at 580 & Fig. 1.

A POSA would be motivated to modify the method of Diaz in view of Forsheiw 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 Diaz in view of Forsheiw to use whole-exome sequencing (WES) rather than whole-genome sequencing (WGS) because Forsheiw teaches that the 10 SNV mutations identified in the breast cancer tumor sample for tracking in cDNA were mutations in gene exons. *See* Forsheiw supplemental materials at Table S7; *see also id.* 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.

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. Forsheiw 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).” Forsheiw at 3.

Forsheiw identifies 40 single-base substitutions (i.e., SNVs) in the ovarian tumor sample. Forsheiw 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 because the whole exome sequences include the SNVs.

To the extent a POSA considering Diaz and its teachings regarding Forsheiw would conclude that Diaz did not achieve the 0.01% sensitivity expressly taught by Diaz, Diaz specifically discloses the advantages of using error-corrected sequencing barcoding strategies to detect SNVs more sensitively. *See* Diaz at 581 (“Newer approaches aimed at increasing overall sensitivity using barcoding strategies in the context of NGS are being investigated. This will be important because false-negative results, especially

when ctDNA results are used for therapeutic decision making or in detecting occult disease, could be detrimental. The absolute sensitivity for ctDNA analysis in a variety of clinical scenarios will have to be evaluated to best understand the limitations and advantages of this new technology.”) Therefore, Diaz provides express motivation to combine the tumor-informed cfDNA assays disclosed in Diaz with barcoding error correction methods. Such a POSA would be motivated to combine Diaz with Young to achieve the claimed sensitivity because both references teach methods for detecting minimal residual disease, and Young teaches an example of the barcoding error correction methods taught by Diaz. *See, e.g.*, Diaz at Table 1 (disclosing applications of liquid biopsy to monitor minimal residual disease), Young at 1611 (“[a] clinical application of ECS is to quantify minimal residual disease (MRD).” Additionally, both references teach tumor-informed assays. Like Diaz, Young involves sequencing tumor samples to identify tumor-specific mutations (*see* Young Supplementary Tables and Figures at Supplementary Tables 2-3), and then designing primers targeting the leukemia-specific variants identified for each patient (*see id.* at Supplementary Table 4). *See also* Young Supplementary Methods at 2 (Study Design). Young describes using multiplex PCR for samples with limited amounts of input DNA. *See id.* at 2-3.

A POSA would further be motivated to combine the teachings of Diaz and Young to arrive at the claimed method because Young discloses that its implementation of error-corrected sequencing “easily targets loci of interest by single or multiplex PCR and inserts seamlessly into the standard NGS library preparation (Supplementary Figure 1, Supplementary Methods).” Young at 1610. Young explains that its ECS Library preparation methods can be implemented by replacing “the Illumina-provided Y-shaped adapters with custom adapters containing a random 16 base pair oligonucleotide index sequence (Supplementary Table 1).” Young Supplementary Materials at 3. Therefore, using the error-corrected sequencing method of Young to identify the SNVs would be a simple combination of prior art elements according to known methods to yield predictable results.

A POSA would also have a reasonable expectation of success because Diaz teaches ctDNA can generally be detected at levels as low as 0.01%, and Young teaches that its error-corrected sequencing (ECS) method “can achieve the 1:10[,000] limit of detection featured by conventional MRD platforms,” including for point substitutions. *See* Young at 1611; *see also id.* at 1610 (disclosing detection of a SNV at 0.000079 (0.0079%) VAF).

A POSA reading Diaz would also be motivated to detect an SNV present in less than or equal to 0.015% of the cell-free DNA having the SNV locus because Diaz discloses that the fraction of circulating DNA contributed from the tumor is sometimes less than 0.015%. *See* Diaz at 580 (“Salient to this review, it is evident that a proportion of fragmented DNA in circulation is derived directly from the tumor. Multiple methods have shown that the fraction of circulating DNA contributed from the tumor varies greatly, between 0.01% and more than 90%.”)

To the extent a POSA considering Diaz including its teachings regarding Forsheu did not achieve the 0.01% sensitivity taught by Diaz, Diaz specifically discloses the advantages of using barcoding strategies such as the strategy disclosed by Kinde in order to detect SNVs more sensitively. *See* Diaz at 580 (“Newer approaches aimed at increasing overall sensitivity using barcoding strategies in the context of NGS are being investigated. This will be important because false-negative results, especially when ctDNA results are used for therapeutic decision making or in detecting occult disease, could be detrimental. The absolute sensitivity for ctDNA analysis in a variety of clinical scenarios will have to be evaluated to best understand the limitations and advantages of this new technology.”). Therefore, Diaz provides express motivation to combine the tumor-informed cfDNA assays disclosed in Diaz with barcoding error correction methods. Such a POSA would be motivated to combine the methods of Diaz with Kinde to achieve the claimed sensitivity because both Diaz and Kinde relate to the field of cancer detection using targeted amplification and next-generation sequencing to detect rare cancer-associated variants, including SNVs, *See* Kinde at 1, and Kinde teaches an example of the barcoding error correction methods taught by Diaz.

In fact, Diaz cites the original Safe-SeqS method (Kinde, I. et al. *Detection and quantification of rare mutations with massively parallel sequencing*. Proc Natl Acad Sci USA. 7;108(23):9530-5 (June 2011) (including its Supplementary Materials, “Kinde 2011”)), a precursor of the Safe-SeqS method disclosed in Kinde, as an example of one such approach for improved sensitivity. such as those disclosed in Kinde 2013. Furthermore, Kinde discloses that its “more sophisticated molecular methods might be able to detect the presence of [cancer mutations] at higher sensitivities and specificities than possible with conventional methods.” Kinde at 2.

A POSA would further have a reasonable expectation of success combining Diaz and Kinde to arrive at the claimed method, because Diaz teaches ctDNA can generally be detected at levels as low as 0.01%, and Kinde teaches that its error correction approach is compatible with multiplex PCR and that “[t]he Safe-SeqS approach used here is capable of detecting 1 mutant template among 5000 to 1,000,000 wild-type templates, depending on the amplicon and the position within the amplicon that is queried.” Kinde at 4. Kinde discloses that relaxed parameters for SNV detection can be used when the SNV to be detected is known *a priori*. *See* Kinde Supplementary Materials at 9. Diaz discloses methods wherein the SNVs to be detected are known *a priori* based on sequencing of a tumor biopsy sample. *See, e.g.,* Diaz at 582 (“[f]uture studies evaluating postoperative levels of ctDNA could offer personalized markers based on the unique mutational profiles of resected tumors and thus having exquisitely high specificity”), 584 (“[o]ne of the key advantages of ctDNA analyses is the high degree of specificity offered, because mutations found in cfDNA are in essence integral agents of an individual’s cancer and are defined by their presence in tumor DNA and absence in matched normal DNA”). Additionally, well before Natera’s asserted priority date, barcoding-based error correction had already been applied to cfDNA to detect SNVs present at levels below 0.015%. *See, e.g.,* Bettgowda C. et al., *Detection of circulating tumor DNA in early- and late-*

stage human malignancies, Sci. Transl. Med. 19;6:224ra24 (Feb. 2014) at 4 (disclosing that Safe-SeqS amplification and sequencing was able to detect one mutant template in the DNA purified from up to 5 ml of plasma)¹.

A POSA reading Diaz would also be motivated to detect an SNV present in less than or equal to 0.015% of the cell-free DNA having the SNV locus because Diaz discloses that the fraction of circulating DNA contributed from the tumor is sometimes less than 0.015%. See Diaz at 580 (“Salient to this review, it is evident that a proportion of fragmented DNA in circulation is derived directly from the tumor. Multiple methods have shown that the fraction of circulating DNA contributed from the tumor varies greatly, between 0.01% and more than 90%.”)

A POSA would also be motivated to combine Diaz and Forsheiw with Patel in order to achieve improved detection of SNVs by increasing sequencing depth and improving the ability to distinguish true SNVs from sporadic errors. See, e.g., Diaz at 581 (“sensitivity is limited by the error rate of DNA polymerase”); Forsheiw at 9 (explaining that Forsheiw 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”); Patel at 1-2 (“Methods as described herein also include detecting and distinguishing true nucleic acid variants from misincorporation errors, sequencer errors, and sample misclassification errors.”). A POSA would therefore be motivated to turn to references such as Patel because such references teach methods for detecting ultra-rare variants with higher read depth or fidelity and/or with improved algorithms. For 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 Diaz and Forsheiw with Patel. For example, Diaz teaches “A mutation profile was determined from each patient’s resected tumor, and this personal and unique molecular signature was then used to create a set of mutation-specific probes for each patient.” Diaz at 582. Forsheiw 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).” Forsheiw at 3. Thus, Diaz and Forsheiw teach that detecting known SNVs using a tumor-informed approach allows SNV

¹ Although Bettgowda uses alternative units to describe the mutant allele fraction, a POSA would have understood that detection of 1 mutant template from 5 ml of plasma corresponds to detection of an SNV present in less than or equal to 0.015% of the cell-free DNA comprising the locus. See, e.g., Horlitz M. et al. *Yields of Viral and Circulating Cell-Free Nucleic Acids Using the QIAamp® Circulating Nucleic Acid Kit* (2011) at 262 (for the cfDNA purification kit used by Bettgowda, explaining that circulating cell-free DNA yields per 5 mL sample varied from about 8,000 to more than 80,000 genome equivalents).

detection with greater sensitivity. Patel teaches a method capable of detecting pre-defined SNVs that are present at less than or equal to 0.015% of the DNA comprising the locus.

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Claim	Diaz																		
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) mutations in the plasma sample, the method comprising:	To the extent the preamble is construed to be limiting, Diaz 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., Diaz at Abstract, Table 1, Fig. 1, Fig. 2, Fig. 3, 579-584. As an example, Diaz Table 1 discloses applications of liquid biopsy including monitoring tumor dynamics, monitoring minimal residual disease, and evaluation of early treatment response: <table><tr><th colspan="2">Table 1. Applications of Liquid Biopsy</th></tr><tr><th colspan="2">Application</th></tr><tr><td>Early detection</td><td></td></tr><tr><td>Assessment of molecular heterogeneity of overall disease</td><td></td></tr><tr><td>Monitoring of tumor dynamics</td><td></td></tr><tr><td>Identification of genetic determinants for targeted therapy</td><td></td></tr><tr><td>Evaluation of early treatment response</td><td></td></tr><tr><td>Monitoring of minimal residual disease</td><td></td></tr><tr><td>Assessment of evolution of resistance in real time</td><td></td></tr></table>	Table 1. Applications of Liquid Biopsy		Application		Early detection		Assessment of molecular heterogeneity of overall disease		Monitoring of tumor dynamics		Identification of genetic determinants for targeted therapy		Evaluation of early treatment response		Monitoring of minimal residual disease		Assessment of evolution of resistance in real time	
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Early detection																			
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Assessment of evolution of resistance in real time																			

EXHIBIT 2-D: NEOGENOMICS' FINAL INVALIDITY CONTENTIONS CLAIM CHARTS
FOR U.S. PATENT NO. 11,530,454
(DIAZ)

U.S. Patent No. 11,530,454	
Diaz	
Claim	
	As another example, Diaz discloses, “Furthermore, blood can be drawn at any time during the course of therapy and allow for dynamic monitoring of molecular changes in the tumor rather than relying on a static time point.” Diaz at 581. As another example, Diaz discloses, “These probes were used to detect and quantify ctDNA after surgery in these patients undergoing potentially curative resection, and the patients were subsequently observed over the course of 2 to 5 years.” Diaz at 583. As another example, Diaz discloses that “[a]nalysis of ctDNA in plasma samples obtained before and after treatment can ultimately provide a global picture of the molecular genetics of a patient’s tumor. This genetic picture includes the dynamic changes in the mutation profile that occur during therapy as well as the heterogeneity that emerges as a result of this therapeutic selective pressure.” Diaz at 583. As another example, Diaz discloses: “Recently, PCR-based digital approaches have been updated with techniques that use next-generation sequencing (NGS) to identify rare mutant variants in complex mixtures of DNA (Table 1). These techniques have expanded the ability to detect a single point mutation, and now multiple genes of interest can be investigated in one sample.” Diaz at 580 (citing Forshew). 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. <i>See, e.g.</i>, 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 7 (“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 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

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U.S. Patent No. 11,530,454 Diaz	
Claim	
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;	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>i.e.</i>, SNVs). It would have been obvious over Diaz to perform whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations. <i>See, e.g.</i>, Diaz at Abstract, 579, 582, 584, Fig. 1, Fig. 3. As an example, Diaz discloses, “Two potential processes have been described for performing ctDNA analysis. The first requires identifying a specific mutation or mutations in the tumor tissue to target and quantify the level of ctDNA at a particular time point.” Diaz at 582. As another example, Diaz discloses, “The best example to date has been in a cohort of patients with colorectal cancer undergoing resection with curative intent. A mutation profile was determined from each patient’s resected tumor, and this personal and unique molecular signature was then used to create a set of mutation-specific probes for each patient. These probes were used to detect and quantify ctDNA after surgery in these patients undergoing potentially curative resection, and the patients were subsequently observed over the course of 2 to 5 years. In this study, ctDNA was shown to be sufficiently sensitive to detect minimal residual disease after surgical resection.” Diaz at 582 (citing Diehl 2008). As another example, Diaz discloses “Future studies evaluating postoperative levels of ctDNA could offer personalized markers based on the unique mutational profiles of resected tumors and thus having exquisitely high specificity.” Diaz at 582. As another example, Diaz discloses “Moreover, recent advances in the sensitivity and accuracy of DNA analysis have allowed for genotyping of cfDNA for somatic genomic alterations found in tumors. The ability to detect and quantify tumor mutations has proven effective in tracking tumor dynamics in real time as well as serving as a

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Diaz	
Claim	liquid biopsy that can be used for a variety of clinical and investigational applications not previously possible.” Diaz at 579 (Abstract). As another example, Diaz discloses ‘Discriminating ctDNA from normal cfDNA is aided by the fact that tumor DNA is defined by the presence of mutations. These somatic mutations, commonly single-base-pair substitutions, are present only in the genomes of cancer cells or precancerous cells and are not present in the DNA of normal cells of the same individual.’ Diaz at 579. As another example, Diaz discloses: “Recently, PCR-based digital approaches have been updated with techniques that use next-generation sequencing (NGS) to identify rare mutant variants in complex mixtures of DNA (Table 1). These techniques have expanded the ability to detect a single point mutation, and now multiple genes of interest can be investigated in one sample.” Diaz at 580 (citing Forshew). A POSA would understand that “identifying a specific mutation or mutations in the tumor tissue” to determine a “mutation profile” from each patient’s resected tumor in Diaz involves sequencing a tumor sample. Mutations such as somatic point mutations are “[c]hanges in the DNA sequence” that are “the most frequent class of variants associated with the development of solid tumors.” Diaz at 583. Diaz further discloses that “next-generation sequencing (NGS)” can be used to identify rare mutant variants in complex mixtures of DNA and that sequencing of tumor tissue is “routine.” <i>Id.</i> at 580. Diaz further discloses that sanger sequencing, pyrosequencing, and next-generation sequencing are all approaches to detect mutations in tumor tissue. <i>Id.</i> at Fig. 1. Furthermore, Diaz teaches that “[m]olecular tools, such as NGS, can be implemented to find genetic differences between the tissue [biopsies] collected before and after therapy.” <i>Id.</i> at 582. Forsheiw 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. <i>See, e.g.,</i> Forshew at 3, 9, 10, Table S3, Table S7.

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Diaz	
Claim	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>i.e.</i>, 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>i.e.</i>, 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:

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
<i>CD1A</i>	chr1:158226710-158226810	chr1:158226762	C	T
<i>BUB1</i>	chr2:111430288-111430374	chr2:111430343	G	A
<i>IQCA1</i>	chr2:237402413-237402499	chr2:237402439	G	A
<i>PIK3CA</i>	chr3:178936048-178936130	chr3:178936091	G	A
<i>ARAP3</i>	chr5:141041694-141041773	chr5:141041739	G	A
<i>MET</i>	chr7:116398593-116398675	chr7:116398635	C	T
<i>GIMAP5</i>	chr7:150439702-150439805	chr7:150439730	C	T
<i>ZFYVE21</i>	chr14:104195437-104195539	chr14:104195486	G	A
<i>KIAA0406</i>	chr20:36641162-36641252	chr20:36641201	C	T
<i>ADAMTS1</i>	chr21:28214239-28214357	chr21:28214274	C	G

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Diaz	
	As another example, Forsheew teaches “We designed a set of 48 primer pairs to amplify 5995 bases of genomic sequence covering coding regions (exons and exon junctions) of <i>TP53</i> and <i>P TEN</i>, and selected regions in <i>EGFR</i>, <i>BR AF</i>, <i>KRAS</i>, and <i>PIK3CA</i> (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).” Forsheew at 3. As another example, Forsheew 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.” Forsheew 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	Diaz in combination with the knowledge of a POSA, 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 the same reaction volume. See, e.g., Diaz at 580, 584, Fig. 1. As an example, Diaz discloses: “Recently, PCR-based digital approaches have been updated with techniques that use next-generation sequencing (NGS) to identify rare mutant variants in complex mixtures of DNA (Table 1).^{37,40-42} These techniques have expanded the ability to detect a single point mutation, and now multiple genes of interest can be investigated in one sample. Amplifications, rearrangements, and aneuploidy may now be detectable as well^{17,41,43-45} (Fig 1).” Diaz at 580. “The analysis of circulating tumor DNA is a promising area of investigation that allows for interrogation of tumor-specific molecular alterations in the circulation.” Diaz at 584.

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Diaz	
Claim 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; and	As another example, Diaz discloses: “One of the key advantages of ctDNA analyses is the high degree of specificity offered, because mutations found in cfDNA are in essence integral agents of an individual’s cancer and are defined by their presence in tumor DNA and absence in matched normal DNA.” Diaz at 584. In view of Diaz and Forshew, it would have been obvious 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. It would have been obvious to amplify 10 to 500 target loci because as Natera has admitted, multiplex amplification of 50 to 100 target loci in a single reaction for detection of mutations in the amplified DNA using high-throughput sequencing was known in the prior art. <i>See, e.g.</i>, United States Patent Application Publication No. 2012/0270212 (published Oct. 25, 2012) (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); [0064] (disclosing “highly efficient highly multiplexed targeted PCR to amplify DNA followed by high throughput sequencing to determine the allele frequencies at each target locus” and stating that “[t]he ability to multiplex more than about 50 or 100 PCR primers in one reaction in a way that most of the resulting sequence reads map to targeted loci is novel and non-obvious”). It would also have been obvious to obtain amplicons having a length of 50-150 bases because Diaz discloses that cfDNA comprises fragments of around 180 base pairs in length. Diaz at 586. A POSA would be motivated to amplify regions that are somewhat shorter than the total length of the cfDNA fragments to allow the primers to bind. 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. <i>See, e.g.</i>, 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

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Claim	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 methods 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; <i>see also</i> 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. <i>See</i> 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. <i>See</i> 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 discloses performing multiplex amplification with a combination of “each forward and reverse target-specific primer” in the same volume. Forshew Supplemental Materials at 2; <i>see also id.</i> at 17-18 (Table S6); Forshew at 6 (Table 2). A POSA would be motivated to modify Diaz in view of Forshew to perform targeted multiplex amplification to amplify 10 to 500 target loci in order to amplify more loci without requiring additional reactions. A POSA would also have a reasonable expectation of success because Forshew discloses performing TAm-Seq with a degree of multiplex of ten or greater. <i>See</i> 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). Similarly, a POSA would be motivated to modify Diaz in view of Forshew to obtain amplicons having a length of 50-150 bases because Diaz discloses that cfDNA comprises fragments of around 180 base pairs in length. Diaz at 586, and Forshew discloses successful amplification of cfDNA targeting amplicons 50-150 bases in length. Kinde also describes a sequencing library approach for detection of rare mutations, which is compatible with either targeted singleplex or targeted multiplex PCR. <i>See</i> Kinde Supplementary Materials at 7-8 (“Assessment of low-frequency mutations”). As another example, Kinde discloses “Templates were prepared for sequencing as described previously (37), with the modifications noted below that facilitated the amplification of multiple gene regions in a single well of a 96-well PCR plate.” Kinde at 7. According to Kinde, “[t]he multiplexed assay was performed in similar fashion utilizing six independent amplifications – each containing 66 ng of DNA (that is, ~400 ng total) – per sample with the primers described in table S5.” Kinde Supplementary Materials at 8; <i>see also</i> Kinde Supplementary Materials at table S5.

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Claim	
	As another example, Kinde discloses Safe-SeqS primers for multiplex amplification in a single reaction volume. Kinde at 5–8. The multiplexed assays of Kinde were performed with “six independent [multiplex] amplifications” using Safe-SeqS primers described in table S5. <i>Id.</i> at 8; see also Kinde supplementary table S5. Because Diaz teaches multiplex PCR wherein “multiple genes of interest can be investigated in one sample” together with NGS to detect mutations in ctDNA (Diaz at 580) and Kinde also teaches Safe-SeqS primers for multiplex PCR, a POSA would have a reasonable expectation of success for 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.
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. <i>Id.</i> 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, Diaz 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. <i>See, e.g.,</i> Diaz at 580, Table 1. As an example, Diaz discloses: “Recently, PCR-based digital approaches have been updated with techniques that use next-generation sequencing (NGS) to identify rare mutant variants in complex mixtures of DNA (Table 1).

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Claim	These techniques have expanded the ability to detect a single point mutation, and now multiple genes of interest can be investigated in one sample.” Diaz at 580. As another example, Diaz discloses: “Newer approaches aimed at increasing overall sensitivity using barcoding strategies in the context of NGS are being investigated.” Diaz at 581. As another example, Diaz discloses: “New approaches using NGS have increased the throughput necessary for detection of chromosomal abnormalities in ctDNA and will open up the possibility of screening for ctDNA as a tool for the early detection of cancer.” Diaz at 584. 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. <i>See, e.g.,</i> 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 (GAllx 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. <i>See</i> Forshew Supplemental Materials at 4 (“De-multiplexing and alignment,” “Mutation identification,” and “Mutation detection”); <i>see also</i> 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.”).

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Claim	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 Forsheiw would have been obvious to a person of ordinary skill in the art. Forsheiw teaches that the multiplex PCR step was designed to avoid unwanted side products. See Forsheiw 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."); Forsheiw 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. 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, Forsheiw 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., Hoffman et al., <i>DNA bar coding and pyrosequencing to identify rare HIV drug resistance mutations</i>, Nucleic Acids Research, 2007, Vol. 35, No. 13, at 3 (teaching that "[g]enomic DNA samples of interest are amplified using primers that include 5' extension providing binding sites for the 454A and B primers" that are required for sequencing); <i>id.</i> at 4, Figure 2 (showing the single amplification step, reproduced below).

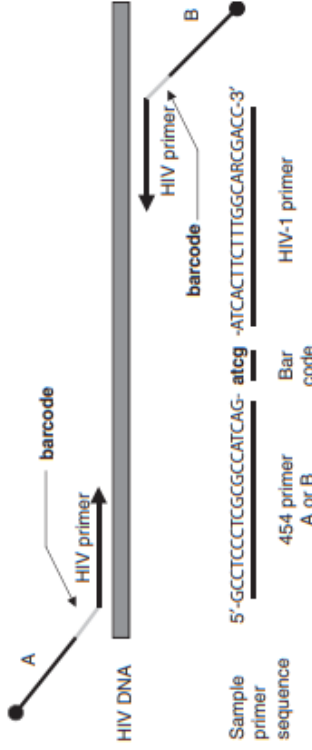
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Claim	 The diagram illustrates the DNA bar coding system. It shows a horizontal line representing HIV DNA. Above the line, a primer labeled 'A' is shown with an arrow pointing right, labeled 'HIV primer'. Below the line, a primer labeled 'B' is shown with an arrow pointing left, labeled 'HIV primer'. Between the primers, a 'barcode' is indicated. Below the barcode, the '454 primer sequence' is given as 5'-GCCTCCCTCGGCCATCAG- atcg -ATCACTTCTTTGGCARGACC-3'. To the right of the barcode, the 'Bar code' is shown as 'HIV-1 primer'. The diagram also shows 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 bulls. Under Natera's apparent interpretation of the Court's adopted construction, Kinde teaches this limitation. See Kinde Supplementary Materials at 8 ("[t]he eluted templates were amplified in a second-round PCR with primers containing the grafting sequences necessary for hybridization to the Illumina GA IIx flow cell at their 5' ends (Fig. 2)"). To the extent Natera argues claim 1 excludes a single plex amplification step between step 1(c)[1] and 1(c)[2], performing Forshe'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. <i>See, e.g.,</i> "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 Forshe 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

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	an intervening single-plex PCR. <i>See</i> “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. <i>See id.</i> 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 (<i>e.g.</i>, automated gel extraction) and would be an obvious modification of Forshew. <i>Id.</i> 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. <i>See, e.g., id.</i>; Thermo Fisher Scientific Inc. <i>Pippin™ DNA size selection systems: Improving NGS Data Everywhere</i> (2013).
1[d]. wherein the sequencing has a depth 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, <i>e.g.</i>, because higher read depth could allow for enhanced detection of lower levels of mutation without change to protocols. <i>See</i> Forshew at 10. 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; <i>see also</i> 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

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Claim	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 (GAllx sequencer, Illumina), with an additional 10 cycles using the barcode sequencing primer, generating ~30 million reads per lane. This produced an average readdepth 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. <i>See</i> 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. <i>See, e.g.</i>, 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). Patel teaches the sequencing has a depth of read of at least 50,000 per target locus. <i>See, e.g.</i>, 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.”).

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Claim 8. The method of claim 1, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation. See, e.g., Forshaw at 2 (performing targeted multiplex amplification to amplify 20 and 48 target loci); Forshaw 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 <i>TP53</i> and <i>PTEN</i>, as well as selected regions in <i>EGFR</i>, <i>BRAF</i>, <i>KRAS</i>, and <i>PIK3CA</i> by overlapping short amplicons); Forshaw at 6 (Table 2); Forshaw Supplemental Materials at 17–18 (Table S6); Forshaw 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., Forshaw 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”); Forshaw 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.)”); Forshaw Supplemental Materials at 2 (“Preamplification methods 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.”); Forshaw 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”). Forshaw 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. Forshaw at Abstract; see also Forshaw at 9 (“Serial plasma samples were collected over the course of 497 days, both before and after	See, e.g., Forshaw at 2 (performing targeted multiplex amplification to amplify 20 and 48 target loci); Forshaw 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 <i>TP53</i> and <i>PTEN</i>, as well as selected regions in <i>EGFR</i>, <i>BRAF</i>, <i>KRAS</i>, and <i>PIK3CA</i> by overlapping short amplicons); Forshaw at 6 (Table 2); Forshaw Supplemental Materials at 17–18 (Table S6); Forshaw 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., Forshaw 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”); Forshaw 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.)”); Forshaw Supplemental Materials at 2 (“Preamplification methods 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.”); Forshaw 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”). Forshaw 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. Forshaw at Abstract; see also Forshaw at 9 (“Serial plasma samples were collected over the course of 497 days, both before and after

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Claim	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.”). Forsheiw also discloses performing TAM-Seq with a degree of multiplex greater than ten. See Forsheiw Fig 1A, Forsheiw Supplemental Materials at 9, Table S1 (disclosing performing TAM-Seq with 48 sequence-specific primer pairs to amplify 5995 baes of genomic sequence). Forsheiw discloses performing multiplex amplification with a combination of “each forward and reverse target-specific primer” in the same volume. Forsheiw Supplemental Materials at 2; see <i>also id.</i> at 17-18 (Table S6); Forsheiw 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 Forsheiw does not disclose this limitation, a POSA would be motivated to modify Forsheiw 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 Forsheiw 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).

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Claim 10. The method of claim 1, wherein the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample.	Forsheiw discloses that the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample. <i>See, e.g.,</i> Forsheiw Supplemental Materials at 2 (“Target-specific primers were designed with universal primer sequences (termed CS1 and CS2) appended at the 5’-end (table S1).”); Forsheiw at 2, Fig. 1 (“Primers were designed to amplify regions of interest in overlapping short amplicons.”); Forsheiw Supplemental Materials at 2 (Primer design for Tam-Seq); Forsheiw at 2 (Figure 1B showing the “initial preamplification step was performed for 15 cycles using a poos of target-specific primer pairs.”). Diaz discloses designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample. <i>See, e.g.,</i> Diaz at 580, 582. As an example, Diaz discloses: “The best example to date has been in a cohort of patients with colorectal cancer undergoing resection with curative intent.¹⁷ A mutation profile was determined from each patient’s resected tumor, and this personal and unique molecular signature was then used to create a set of mutation-specific probes for each patient. These probes were used to detect and quantify ctDNA after surgery in these patients undergoing potentially curative resection, and the patients were subsequently observed over the course of 2 to 5 years.” Diaz at 582. As another example, Diaz discloses: “Recently, PCR-based digital approaches have been updated with techniques that use next-generation sequencing (NGS) to identify rare mutant variants in complex mixtures of DNA (Table 1). These techniques have expanded the ability to detect a single point mutation, and now multiple genes of interest can be investigated in one sample.” Diaz at 580. A POSA would understand that PCR involves designing primers to the target loci. Kinde also describes designing PCR primers targeting the plurality of SNV mutations identified in the tumor sample. <i>See, e.g.,</i> Kinde Supplementary Materials at 7-8

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Claim	
	Kinde also describes a sequencing library approach for detection of rare mutations, which is compatible with either targeted singleplex or targeted multiplex PCR. See Kinde Supplementary Materials at 7-8 (“Assessment of low-frequency mutations”). As another example, Kinde discloses “Templates were prepared for sequencing as described previously (37), with the modifications noted below that facilitated the amplification of multiple gene regions in a single well of a 96-well PCR plate.” Kinde at 7. According to Kinde, “[t]he multiplexed assay was performed in similar fashion utilizing six independent amplifications – each containing 66 ng of DNA (that is, ~400 ng total) – per sample with the primers described in table S5.” Kinde Supplementary Materials at 8; see also Kinde Supplementary Materials at table S5. As another example, Kinde discloses Safe-SeqS primers for multiplex amplification in a single reaction volume. Kinde at 5–8. The multiplexed assays of Kinde were performed with “six independent [multiplex] amplifications” using Safe-SeqS primers described in table S5. <i>Id.</i> at 8; see also Kinde supplementary table S5.
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.	Forsheiw 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., Forsheiw 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.”); Forsheiw 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 <i>EGFR</i> (exon 21), at AF of 6% (patient 27, Table 1).”); Forsheiw 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%.”).

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Claim mutations detected in the cell-free DNA.	Diaz also discloses that the method further comprises detecting recurrence and/or metastases of the cancer from the SNV mutations detected in the cell-free DNA. <i>See, e.g.,</i> Diaz at 582, Table 1, Fig. 3.) Diaz teaches in an example that “[a]ll patients with detectable postoperative levels of ctDNA experienced recurrence, whereas all patients with undetectable postoperative levels of ctDNA remained disease free.” (<i>Id.</i> Diaz at 582.) Diaz further teaches that studies “evaluating postoperative levels of ctDNA could offer personalized markers based on the unique mutational profiles of resected tumors and thus having exquisitely high specificity” which will “rely on the ability to detect low levels of ctDNA released from <i>micrometastatic deposits</i> not detectable by imaging or other diagnostic modalities.” (<i>Id.</i> at 582 (emphasis added); <i>see also id.</i> at Fig. 3 (teaching a method comprising monitoring for metastatic mutations in plasma DNA.) Furthermore, a POSA would understand that applications of the cfDNA assay such as minimal residual disease monitoring described by Diaz include detecting recurrence.
14[pre]. A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in	To the extent the preamble is construed to be limiting, Forsheew 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. <i>See</i> claim 1[pre] above. To the extent the preamble is construed to be limiting, Diaz also 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. <i>See</i> claim 1[pre] above.

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the plasma sample, the method comprising:	
14[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. 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. See claim 1[b] above. Diaz, alone or in combination with a POSA, also discloses performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA	

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Claim 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[pre] above.	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.
14[c]. sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV	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. <i>Id.</i> 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.

EXHIBIT 2-D: NEOGENOMICS' FINAL INVALIDITY CONTENTIONS CLAIM CHARTS
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Claim mutations present in the cell-free DNA from the sequence reads,	Under Natera's apparent interpretation of the Court's adopted construction, Diaz 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 claim 1[c] above. Under Natera's apparent interpretation of the Court's adopted construction, Forsheew also 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 claim 1[c] above. Under Natera's apparent interpretation of the Court's adopted construction, Kinde also 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 Forsheew'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 Forsheew 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 <i>id.</i>

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Claim	
	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 (<i>e.g.</i>, automated gel extraction) and would be an obvious modification of Forshew. <i>Id.</i> 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. <i>See, e.g., id.</i>; Thermo Fisher Scientific Inc. <i>Pippin™ DNA size selection systems: Improving NGS Data Everywhere</i> (2013). <i>See claim 1[c] above.</i>
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.	Diaz discloses that 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. <i>See, e.g., Diaz at 580, Fig. 1.</i> As an example, Diaz discloses: “Technically, sensitivity is limited by the error rate of DNA polymerase, which is generally considered to be 0.01%. Therefore, if the fraction of ctDNA in a sample is at or below 0.01%, it is considered negative for ctDNA.” Diaz at 580. As another example, Diaz discloses: “Newer approaches aimed at increasing overall sensitivity using barcoding strategies in the context of NGS are being investigated. This will be important because false-negative results, especially when ctDNA results are used for therapeutic decision making or in detecting occult disease, could be detrimental. The absolute sensitivity for ctDNA analysis in a variety of clinical scenarios will have to be evaluated to best understand the limitations and advantages of this new technology.” Diaz at 580 (citing Kinde 2011). Young discloses one such barcoding strategy to increase overall sensitivity in the context of NGS. <i>See, e.g., Young at 1608-1611, Table 1; Young Supplementary Tables and Figures at Supplementary Figures 2 and 3; Young Supplementary Methods at 5.</i>

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Claim	As another example, Diaz discloses: “Recently, PCR-based digital approaches have been updated with techniques that use next-generation sequencing (NGS) to identify rare mutant variants in complex mixtures of DNA (Table 1). These techniques have expanded the ability to detect a single point mutation, and now multiple genes of interest can be investigated in one sample.” Diaz at 580. A POSA reading Diaz would be motivated to detect an SNV present in less than or equal to 0.015% of the cell-free DNA having the SNV locus because Diaz discloses that the fraction of circulating DNA contributed from the tumor is sometimes less than 0.015%. <i>See</i> Diaz at 580 (“Salient to this review, it is evident that a proportion of fragmented DNA in circulation is derived directly from the tumor. Multiple methods have shown that the fraction of circulating DNA contributed from the tumor varies greatly, between 0.01% and more than 90%.”) As another example, Fig. 1 of Diaz discloses that Tam-Seq is one example of a method that achieves the sensitivity to detect rare variants present at 0.01% or lower:

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Claim	<table><tr><th>Technique</th><th>Sensitivity</th><th>Optimal Application</th></tr><tr><td>Sanger sequencing</td><td>> 10%</td><td>Tumor tissue</td></tr><tr><td>Pyrosequencing</td><td>10%</td><td>Tumor tissue</td></tr><tr><td>Next-generation sequencing</td><td>2%</td><td>Tumor tissue</td></tr><tr><td>Quantitative PCR</td><td>1%</td><td>Tumor tissue</td></tr><tr><td>ARMS</td><td>0.10%</td><td>Tumor tissue</td></tr><tr><td>BEAMing, PAP, Digital PCR, TAM-Seq</td><td>0.01% or lower</td><td>ctDNA, rare variants in tumor tissue</td></tr></table>			Technique	Sensitivity	Optimal Application	Sanger sequencing	> 10%	Tumor tissue	Pyrosequencing	10%	Tumor tissue	Next-generation sequencing	2%	Tumor tissue	Quantitative PCR	1%	Tumor tissue	ARMS	0.10%	Tumor tissue	BEAMing, PAP, Digital PCR, TAM-Seq	0.01% or lower	ctDNA, rare variants in tumor tissue
Technique	Sensitivity	Optimal Application																						
Sanger sequencing	> 10%	Tumor tissue																						
Pyrosequencing	10%	Tumor tissue																						
Next-generation sequencing	2%	Tumor tissue																						
Quantitative PCR	1%	Tumor tissue																						
ARMS	0.10%	Tumor tissue																						
BEAMing, PAP, Digital PCR, TAM-Seq	0.01% or lower	ctDNA, rare variants in tumor tissue																						

Young also discloses a method for detecting an “ultra-rare” SNV that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus.

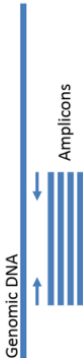
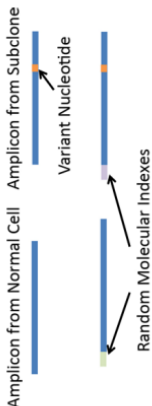
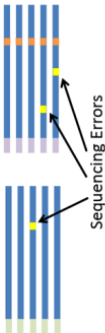
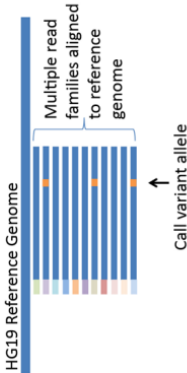
See, e.g., Young at 1608-1611, Table 1; Young Supplementary Tables and Figures at Supplementary Figures 2 and 3; Young Supplementary Methods at 5 (“we were 99% specific to detect variants above... 0.000079 VAF [0.0079% VAF] for the other eight possible substitutions [other than G to T (C to A) or C to T (G to A)]”). As an example, Young discloses: “Here we present a direct application of error-corrected sequencing (ECS) to study clonal heterogeneity during leukemogenesis and validate the accuracy of this method with a series of benchmarking experiments.” Young at 1608, 1610.

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Claim	
	As another example, Young discloses: “We employed ECS by tagging individual DNA molecules with adapters containing 16 bp random oligonucleotide molecular indexes in a manner similar to other reports. Our implementation of ECS easily targets loci of interest by single or multiplex PCR and inserts seamlessly into the standard NGS library preparation (Supplementary Figure 1, Supplementary Methods).” Young at 1610. As another example, Young discloses: “Our only deviations from the standard protocol are ligation of customized adapters containing random indexes instead of the manufacturer’s supplied adapters and a quantitative PCR (qPCR) quantification step before sequencing (Supplementary Table 1). Following sequencing, sequence reads containing the same index and originating from the same molecule are grouped into read families. Sequencing errors are identified by comparing reads within a read family and removed to create an error-corrected consensus sequence (ECCS).” Young at 1610. As another example, Young discloses that “[a] clinical application of ECS is to quantify minimal residual disease (MRD).” Young at 1611. As another example, Young discloses that “[t]he results demonstrate that ECS is quantitative to a VAF of 1:10,000 molecules and provides a highly reproducible digital readout of tumor DNA prevalence in a heterogeneous DNA sample (r2 of 0.9999 and 0.9991, Figures 1a and b).” Young at 1610. As another example, Young discloses that “[w]hen separated by substitution type, variants identified from the ECCSs were 99% specific at a VAF of 0.0034 for G to T (C to A) mutations, 0.00020 for C to T (G to A) mutations and 0.000079 for the other eight possible substitutions.” Young at 1610. As another example, Young discloses: “Leveraging this information, conventional NGS was implemented retrospectively to detect MRD harboring leukemia-specific insertions/deletions (indels) as rare as 0.00001 VAF in NPM111 and 0.0001 VAF in RUNX1. This was possible because indels are only rarely generated erroneously by NGS. Unfortunately, measuring rare leukemia-associated substitutions is limited owing to the relatively high error profile of conventional NGS. However, ECS can achieve the 1:10,000 limit of detection featured by conventional MRD platforms. For patients whose leukemia lacks suitable markers for conventional MRD, ECS

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Claim	could offer an alternative with comparable sensitivity and specificity that is easy to implement in a clinical sequencing lab.” Young at 1611. As another example, Young Supplementary Figure 1 illustrates the simple workflow for error-corrected sequencing to detect true SNVs, which is compatible with single-plex PCR or multiplex PCR:

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Claim	Supplementary Figure 1. Error-corrected sequencing workflow. Schematic depiction of library preparation and bioinformatics analysis for generating read families and error-corrected consensus sequences. Library Preparation and Sequencing a. Amplify region of interest from genomic DNA  b. Prepare sequencing library  c. Sequence library  Bioinformatics Analysis d. Generate read families  e. Create error-corrected consensus-sequence (ECCS)  f. Align ECCSs 	

Young Supplementary Tables and Figures at Supplementary Figure 1; see also Young at 1610.

Young Supplementary Tables and Figures at Supplementary Figure 1; *see also* Young at 1610.

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Claim	Kinde also discloses a method for detecting SNVs present at less than or equal to 0.015% for cancer detection. Kinde sought to develop a method of assaying for somatic cancer mutations in samples wherein “the amount of DNA derived from neoplastic cells... would be relatively small compared to the DNA derived from normal cells.” Kinde at 3-4. According to Kinde, “[t]his necessitated the use of an analytic technique that could reliably identify a rare population of mutant alleles among a great excess of wild-type alleles. A modification of one of the Safe-SeqS (Safe-Sequencing System) procedures described in (37), in which DNA templates are amplified with modified gene-specific primers, was designed for this purpose (Fig. 2).” Kinde at 4. Kinde discloses a Safe-Seq approach using gene-specific primers comprising unique identifiers (UIDs) for targeted amplification, followed by further universal amplification. See Kinde at 4-5, Fig. 2. According to Kinde, “[t]he Safe-SeqS approach used here is capable of detecting 1 mutant template among 5000 to 1,000,000 wild-type templates, depending on the amplicon and the position within the amplicon that is queried.” Kinde at 4. Kinde demonstrated detection of SNVs wherein “[t]he median fraction of mutant alleles was 3% and ranged from 0.01 to 80% (Fig. 3 and table S3).” Kinde at 4; see also FIG. 3 and Kinde Supplementary Materials at table S3. Kinde further explains that “[a]mplifications of DNA from nonneoplastic tissues were used as negative controls in these experiments to define the detection limits of each queried mutation. In all cases, the fraction of mutant alleles was significantly different from the background mutation levels in the negative controls ($P < 0.001$, binomial test).” Id. As an example, as shown in Kinde’s supplemental Table S3, the mutation detected at 0.01% in the PAP 026 sample was an SNV (a single base substitution from G to A, described in genomic coordinates as g.chr10:89710752G>A). Kinde supplementary Table S3. Kinde further explains that the modified Safe-SeqS assay is compatible with multiplex PCR, and designed such an assay based on multiplex PCR. See Kinde at 5, Fig. 2. Kinde demonstrated application of the Safe-SeqS assay

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Claim	with multiplex PCR as a screening test to target regions from commonly mutated cancer genes. Id. According to Kinde, the 46 amplicons of the screening test covered 10,257 bp, and “[g]iven the accuracy of Safe-Seq, this number was adequate to comfortably detect mutations existing in >0.1% of template molecules.” Kinde at 5. Kinde explains that they used a different analytical approach to detect mutations in the context of the screening assay, because in the screening assay mutations were not known a priori. See Kinde Supplementary Materials at 9. Specifically, Kinde discloses: “For the 46 assays querying a single amplicon, we required that the fraction of mutant alleles was significantly different from the background mutation levels determined from a negative control (P < 0.001, binomial test). As mutations are not known a priori in a screening environment, we used a more agnostic metric to detect mutations in the multiplexed assay. A threshold supermutant frequency was defined for each sample as the sum of the mean frequency of supermutants – including only those with average quality scores ≥ 25 and excluding those at positions with relatively high-frequency variants (such as SNPs or any mutation present at proportions $\geq 1\%$) – and six standard deviations of the mean. Only supermutants exceeding this threshold and representing at least two template molecules were designated as mutations and reported in Fig. 4 and table S6.” Kinde Supplementary Materials at 9. Therefore, a POSA would understand that the higher variant allele fraction of 0.1% chosen by Kinde to “comfortably detect” mutations in the screening assay would not be required for a multiplex assay where the mutations to be detected were known a priori. A POSA would have a reasonable expectation of success for detecting a SNV mutation that is present in less than or equal to 0.015% of sample DNA having the SNV locus because Kinde teaches that Safe-SeqS is “capable of detecting 1 mutant template among 5000 to 1,000,000 wild-type templates, depending on the amplicon and the position within the amplicon that is queried,” and that Safe-SeqS is compatible with multiplex PCR. See Kinde at 5, Fig. 2, Kinde Supplementary Materials at 7-8. A POSA would further have a reasonable expectation of success because Kinde teaches that the more relaxed SNV detection parameters can be used when mutations to be detected are known a priori rather than in an agnostic screening context. Kinde Supplementary Materials at 9.

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Claim	
	Forsheiw 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., Forsheiw at 3 (“Background frequencies of nonreference reads were ~0.1% (median, 0.03%; mean, 0.2%; in keeping with Q30 quality threshold applied”); Forsheiw 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); Forsheiw at 9 (“Enhanced detection down to 1 mutant allele in 10,000 or more wild-type alleles can be obtained.”); Forsheiw Supplemental Materials at 17-19, Tables S6—S7 (identification of tumor mutations that are single nucleotide changes or substitutions in a genome, <i>i.e.</i>, SNVs). Under Natera’s interpretation of the claim, Forsheiw 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, Forsheiw also teaches the 0.015% limitation because Forsheiw teaches background error rates for SNVs that are below 0.015% (see, e.g., Forsheiw 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. Forsheiw 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).” Forsheiw at 3. As another example, Forsheiw discloses that “Amplicon libraries were tagged and pooled together for sequencing with libraries prepared from 24 control samples.” Forsheiw at 4. In the context of screening for mutations, Forsheiw 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

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Claim	conservation (Fig. 1D).” ForsheW at 3. In that context, ForsheW discloses “we obtained 100% sensitivity, identifying mutations at AFs as low as 0.6%.” <i>Id.</i> 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).” <i>Id.</i> ForsheW further discloses 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.” <i>Id.</i> 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).” <i>Id.</i> 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. <i>See, e.g., Metzker 2/11/2025 Dep. Tr. at 79:8-23</i> (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 detectable frequencies would be much lower without a requirement for 90% confidence. <i>See</i> 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:

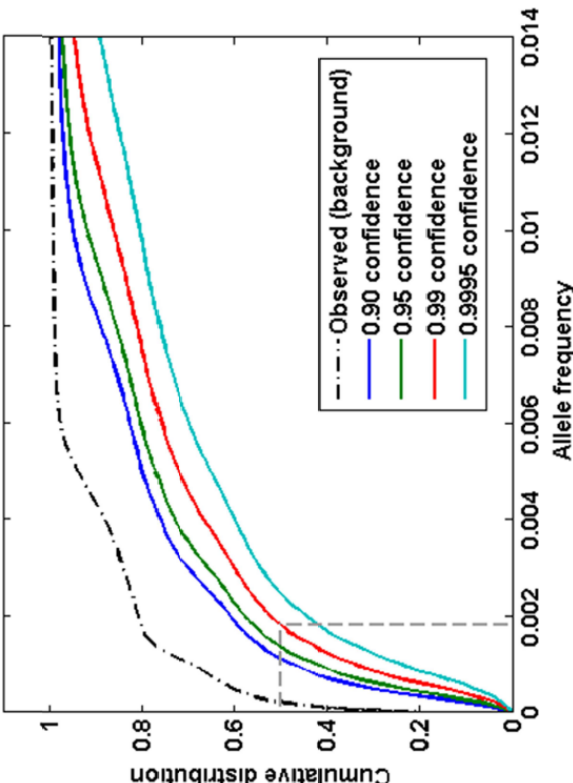
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	 Forsheiw supplementary materials at Figure S3. According to Dr. Metzker’s analysis, Forsheiw’s method could detect mutations that are present at or above the level of background noise in an assay. <u>See, e.g., Metzker 11/14/2023 Dep. Tr. at 98:11-101:13 (using “brown M&Ms” as an analogy for noise and “red M&Ms” as an analogy for ctDNA).</u> Therefore, under Natera’s apparent interpretation of this limitation, Forsheiw’s 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%. <u>See Forsheiw Figure S3.</u> As another example, Forsheiw 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

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Claim	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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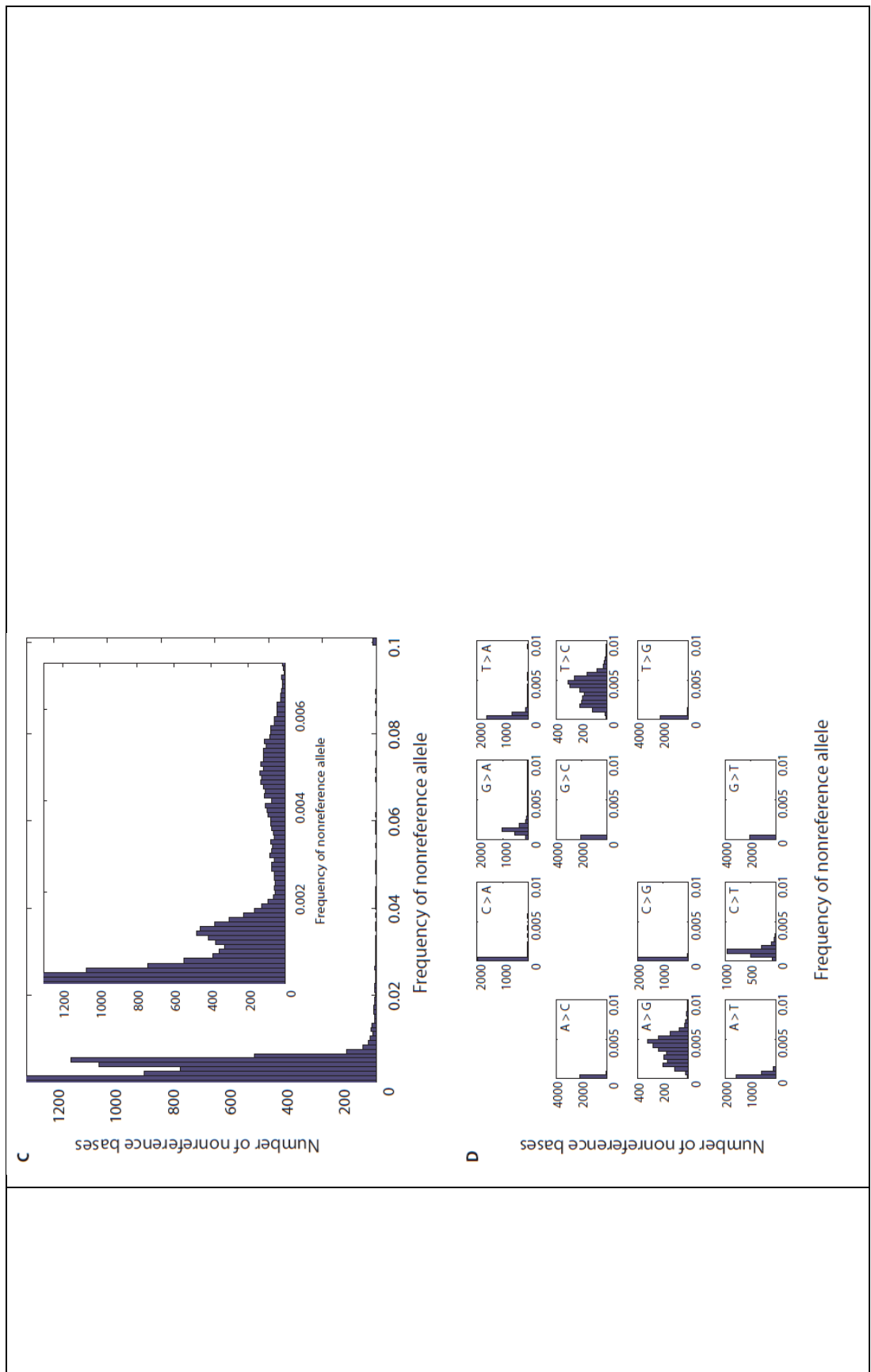
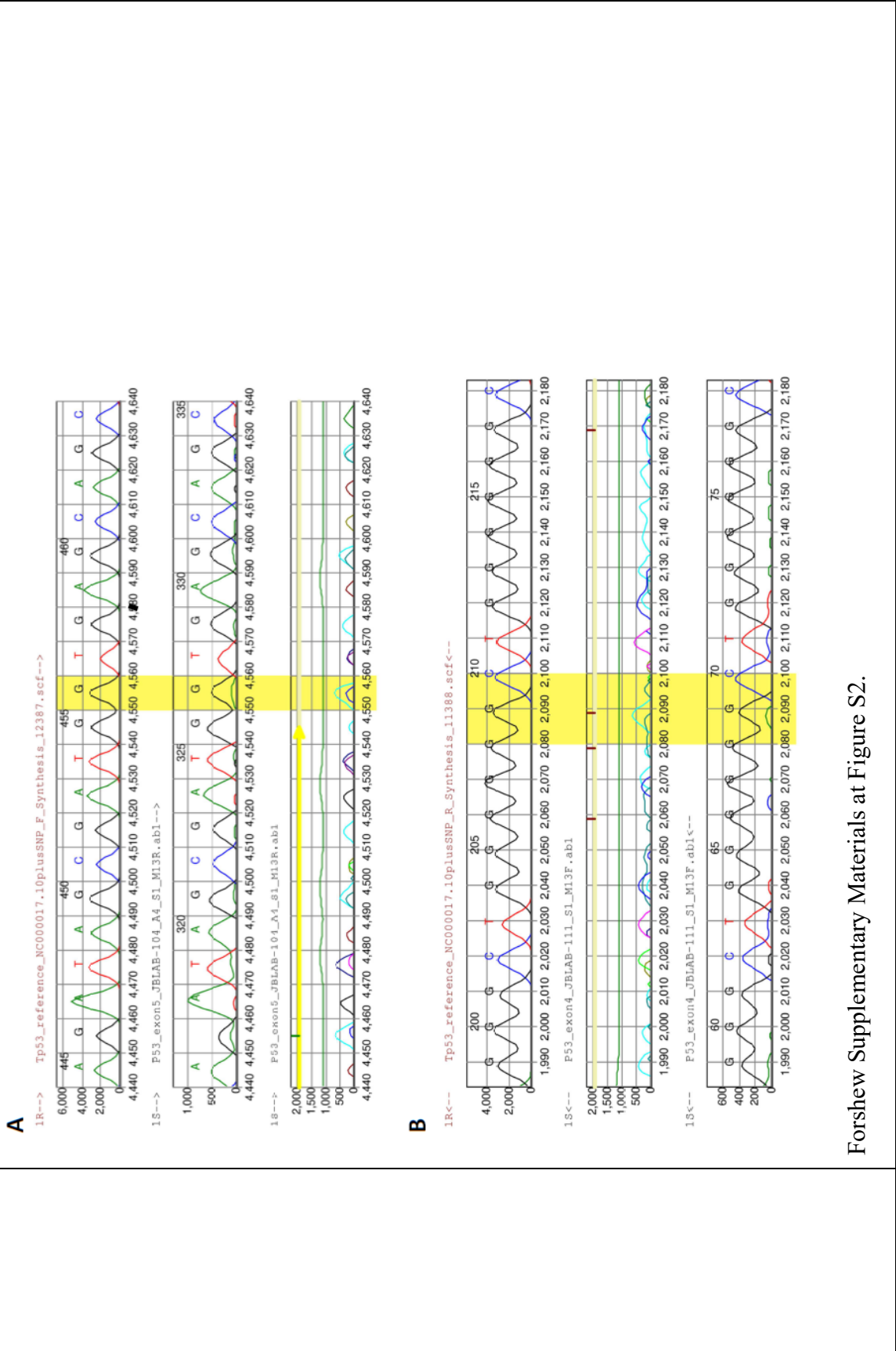


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Claim	
	As another example:

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Forsheiw Supplementary Materials at Figure S2.

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Claim	Forshew teaches that identifying patient-specific mutations comprises sequencing matched tumor and normal tissue samples from a patient. <i>See</i> 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).” <i>Id.</i> As another example, Forshew discloses 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. 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.

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Claim	
	As another example, Forsheew 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.” As another example, Forsheew discloses that “[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols.” NEOGEN00275326 and NEOGEN00275329 provide further evidence of motivation and reasonable expectation of success for increased read depths. <i>See, e.g.</i>, 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 Forsheew specifically teaches, “[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols.” Forsheew at 10. <i>See also</i> Forsheew 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%.”).
15. The method of claim 14, wherein the sequencing has a depth of read	Forsheew discloses the sequencing has a depth of read of at least 50,000 per target locus. <i>See also</i> claim 1[d] and 14[b], [c], [d] above.

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Claim	
of at least 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.	Forsheew discloses that the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation. <i>See claim 8 above.</i>
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.	Forsheew discloses that the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample. <i>See claim 10 above.</i> Diaz, alone or in combination with the knowledge of a POSA, also discloses that the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample.

EXHIBIT 2-D: NEOGENOMICS' FINAL INVALIDITY CONTENTIONS CLAIM CHARTS
FOR U.S. PATENT NO. 11,530,454
(DIAZ)

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targeting the plurality of SNV mutations identified in the tumor sample.	See claim 10 above. Kinde also 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 metastases of the cancer from the SNV mutations detected in the cell-free DNA. See claim 12 above.	Forsheew 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.
28. The method of claim 14, wherein the method is	Forsheew 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.

EXHIBIT 2-D: NEOGENOMICS’ FINAL INVALIDITY CONTENTIONS CLAIM CHARTS
FOR U.S. PATENT NO. 11,530,454
(DIAZ)

U.S. Patent No. 11,530,454	
Diaz	
Claim	
capable of detecting an SNV mutation that is present in 0.005% to 0.015% of the cell-free DNA comprising the SNV locus.	