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

Reference: Diaz, LA Jr. and A. Bardelli, *Liquid Biopsies: Genotyping Circulating Tumor DNA*, J. Clin. Oncol. 20;32(6):579-86 (Feb. 20, 2014) (Epub 2014 Jan 21) ("Diaz").

To the extent Natera asserts that Diaz does not disclose or teach one or more limitations of asserted claims 1, 11, 12, or 18, performing the methods as claimed would have been obvious to a person of ordinary skill in the art at the time of the alleged invention considering Diaz in combination with the knowledge of a POSA and/or 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: Forshew, T. et al., *Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA*, 4(136):136RA68 (May 30, 2012) ("Forshew")

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

A POSA would have considered the disclosures of the aforementioned prior art because the prior art references all concern the same subject matter, namely amplification and/or sequencing of DNA for the purpose of identifying mutations therein, and particularly detection of rare SNVs in a wild-type background.

A POSA would also have been motivated to combine Diaz with Forshew and/or Bashashati because all of the references relate to the same field, namely, detection of mutations in ctDNA.

A POSA would also have been motivated to apply the teachings of Forshew 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

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mutation, and now multiple genes of interest can be investigated in one sample." Diaz at 580. Diaz expressly cites Forshew for this teaching, and Forshew provides one such assay. A POSA would have had a reasonable expectation of successfully considering Forshew's teaching in the context of Diaz because Diaz expressly teaches that Forshew'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 Forshew'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 Forshew to use whole-exome sequencing (WES) rather than whole-genome sequencing (WGS) to identify the tumor-specific mutations in the tumor biopsy sample because WES is cheaper than WGS.

A POSA would also have a reasonable expectation of success in modifying the method of Diaz in view of Forshew to use whole-exome sequencing (WES) rather than whole-genome sequencing (WGS) because Forshew teaches that the 10 SNV mutations identified in the breast cancer tumor sample for tracking in cfDNA were mutations in gene exons. *See* Forshew 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. Forshew further discloses that "43 single-base substitutions were called (table S3)" and "[t]hese matched 100% of mutations identified by Sanger sequencing and included three additional mutations at low [allele frequencies] that were below detection thresholds of Sanger sequencing (fig. S2)." Forshew at 3.

Forshew identifies 40 single-base substitutions (i.e., SNVs) in the ovarian tumor sample. Forshew supplemental materials at 13–14, Table S3. These "single-base substitutions" are tumor-specific SNVs, and a POSA would have understood that such tumor-specific SNVs could be identified in the same manner by performing whole exome sequencing because the whole exome sequences include the SNVs.

To the extent a POSA considering Diaz and its teachings regarding Forshew would conclude that Diaz 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

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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 it's ECS Library preparation methods can be implements by replacing "the Illumina-provided Y-shaped adapters with custom adapters containing a random 16 base pair oligonucleotide index sequence (Supplementary Table 1)." Young Suppl. 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%.")

A POSA would have been motivated to combine the methods of Diaz, Young, and Bashashati to use whole-exome sequencing in order to determine the mutations in the tumor biopsy, because all three references relate to tumor-informed approaches to detecting

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cancer mutations in liquid biopsies, and Bashashati teaches that whole-exome sequencing of tumor samples is a suitable approach to identify tumor-specific identify mutations for profiling (*see, e.g.,* Bashashati supplementary app'x at 5–6; Bashashati at 22), as described by Diaz (*see, e.g.,* Diaz at 582, 584). The prior art teaches that whole-exome sequencing and whole genome sequencing are known alternative for each other. United States Patent Application Publication No. US 2016/0032396 ("Diehn") at [0270]–[0271] ("The method may comprise (a) obtaining a genotype of a tumor in a subject; (b) identifying genomic regions comprising one or more mutations based on the genotype of the tumor; and (c) producing a selector set comprising at least one genomic region. Obtaining the genotype of the tumor in the subject may comprise conducting a sequencing reaction on a sample from the subject. Sequencing may comprise whole genome sequencing. Sequencing may comprise whole exome sequencing.") Therefore, using whole exome sequencing to identify the SNVs would be a simple combination of prior art elements according to known methods to yield predictable results.

To the extent a POSA considering Diaz including its teachings regarding Forshew 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 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 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. 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

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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.,* Bettegowda C. et al., *Detection of circulating tumor DNA in early- and late-stage human malignancies*, Sci Transl Med. 19;6(224):224ra24 (Feb. 2014) ("Bettegowda"), 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 have been motivated to combine the methods of Diaz, Kinde, and Bashashati to use whole-exome sequencing in order to determine the mutations in the tumor biopsy, because all three references relate to tumor-informed approaches to detecting cancer mutations in liquid biopsies, and Bashashati teaches that whole-exome sequencing of tumor samples is a suitable approach to identify tumor-specific identify mutations for profiling (*see, e.g.,* Bashashati supplementary app'x at 5–6; Bashashati at 22), as described by Diaz (*see, e.g.,* Diaz at 582, 584). Additionally, Diaz cites methods such as TAm-Seq (Forshew 2012) which similarly used whole exome sequencing to identify SNVs in a tumor biopsy before performing a tumor-informed cfDNA assay. Therefore, using whole exome sequencing to identify the SNVs would be a simple combination of prior art elements according to known methods to yield predictable results. The prior art teaches that whole-exome sequencing and whole genome sequencing are known alternatives for each other. Diehn at [0270]–[0271] ("The method may comprise (a) obtaining a genotype of a tumor in a subject; (b) identifying genomic regions comprising one or more mutations based on the genotype of the tumor; and (c) producing a selector set comprising at least one

[1] Although Bettegowda 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 Bettegowda, explaining that circulating cell-free DNA yields per 5 mL sample varied from about 8,000 to more than 80,000 genome equivalents).

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genomic region. Obtaining the genotype of the tumor in the subject may comprise conducting a sequencing reaction on a sample from the subject. Sequencing may comprise whole genome sequencing. Sequencing may comprise whole exome sequencing.")

A POSA would be motivated to combine the method of Diaz and Bashashati in order to identify the tumor-specific mutations that provide the "personalized markers based on the unique mutational profiles of resected tumors" that therefore have "exquisitely high specificity," as taught by Diaz at 582. Additionally, a POSA would have a reasonable expectation of success because Bashashati successfully identified tumor-specific SNVs using WES. *See, e.g.,* Bashashati supplementary app'x at 5–6; Bashashati at 22.

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

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	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 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 582. 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. Forshew also discloses a method for preparing biological samples useful for monitoring the progression of cancer in a subject. *See, e.g.,* Forshew at 1 (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

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	sequencing (TAm-Seq)] to noninvasively identify the origin of metastatic relapse in a patient with multiple primary tumors."); *id.* at 9 ("Whole-genome sequencing of tumor material was used to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy. Ten mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci . . . Serial plasma samples were collected over the the course of 497 days, both before and after treatment. We performed TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and tracked dynamics of all mutations in parallel[.]"); *id.* at 4 ("We applied TAm-Seq to directly identify mutations in plasma of cancer patients."); Forshew supplemental materials at 1 ("Dilution of plasma DNA samples to determine allele quantification accuracy"); *id.* at 17-19, Tables S6-S7 (identification of tumor mutations that are single nucleotide changes or substitutions in a genome, *i.e.,* SNVs).
1(a). performing sequencing on a tumor biopsy sample of the subject to identify a plurality of tumor-specific mutations, wherein the tumor-specific mutations comprise one or more single nucleotide variant (SNV) mutations;	Diaz discloses performing sequencing on a tumor biopsy sample of the subject to identify a plurality of tumor-specific mutations, wherein the tumor-specific mutations comprise one or more single nucleotide variant (SNV) mutations. *See, e.g.,* 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

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	sufficiently sensitive to detect minimal residual disease after surgical resection." Diaz at 582 (citing Diehl, F. et al.: *Circulating mutant DNA to assess tumor dynamics*, Nat'l Med. 13:985-990, (2008) ("Diehl")). 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 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 biopsy. Diaz at 582-83. 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[.]" *Id.* at 583. Diaz further discloses that "next-generation sequencing (NGS)" can be used to

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	identify rare mutant variants in complex mixtures of DNA and that sequencing of tumor tissue is "routine." *Id.* at 580. Diaz further discloses that sanger sequencing, pyrosequencing, and next-generation sequencing are all approaches to detect mutations in tumor tissue. *Id.* 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." *Id.* at 582. Kinde also discloses performing sequencing on a tumor biopsy sample of the subject to identify a plurality of tumor-specific mutations, wherein the tumor-specific mutations comprise one or more single nucleotide variant (SNV) mutations. *See*, *e.g.,* Kinde at 5 (discloses multiplexed analysis of a plurality of tumor-specific mutations ("This multiplexed approach included 50 primer pairs that amplified segments of 241 to 296 base pairs (bp) containing frequently mutated regions of DNA. The regions to be amplified were chosen from the most commonly mutated genes in endometrial and ovarian cancers (Table 2)[.]")); *id.* at 3, Table 2, Table S2 (discloses these mutations as being somatic mutations ("A complete list of the 12,795 somatic mutations identified in the 22 cancers is provided in table S2. The most commonly mutated genes included the phosphatidylinositol 3-kinase pathway genes PTEN and PIK3CA (34), the adenomatous polyposis coli pathway genes APC and CTNNB1, the fibroblast growth factor receptor FGFR2, the adapter protein FBXW7, and the chromatin-modifying genes ARID1A and MLL2 (Table 2). Genes in these pathways were mutated in both group N and H tumors. Our results are consistent with previous studies of endometrioid endometrial cancer that had evaluated small numbers of genes, although mutations in FBXW7, MLL2, and APC had not been appreciated to occur as frequently as we found them. It was also interesting that few TP53 mutations (5%) were found in these endometrial cancers (Table 2 and table S2), a finding also consistent with previous studies[.]")). A POSA would recognize these mutations as qualifying as tumor-specific mutations, and would readily understand that these can comprise SNVs. *See* Kinde at 6 (discloses the use of DNA derived from cells or cell fragments from endometrial and/or ovarian cancers as a source of DNA for analysis of tumor-specific mutations (*e.g.,* SNVs)

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	("diagnostically useful amounts of cells or cell fragments from endometrial and ovarian cancers are present in the cervix and can be detected through molecular genetic approaches. . . [t]hese findings, in conjunction with technical advances allowing the reliable detection of mutations present in only a very small fraction of DNA templates, are the foundations of the PapGene test")). *See* Kinde at 7 (specifies a tumor biopsy as one potential method of obtaining the tumor DNA to be analyzed ("Development of an improved collection method may also be important to improve sensitivity. . . A small cannula introduced into the endometrial cavity, similar to the Pipelle endometrial biopsy instrument, could theoretically be used to obtain a more highly enriched sample of cells coming from the endometrium, fallopian tube, and ovary[.]")). Forshew also discloses performing sequencing on a tumor biopsy sample of the subject to identify a plurality of tumor-specific mutations, wherein the tumor-specific mutations comprise one or more single nucleotide variant (SNV) mutations. *See, e.g.,* Forshew at 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"); *id.* at 3 ("We therefore applied a two-step amplification process: a limited-cycle preamplification step where all primer sets were used together to capture the starting molecules present in the template, followed by individual amplification to purify and select for intended targets. (Fig. 1B) (Supplementary Methods)."); Forshew supplemental materials at 2 ("Preamplification reactions were carried out in 10-µl reaction volumes containing 50 nM of each forward and reverse target-specific primer . . . Reactions were subjected to 15 cycles of amplification[.]"); Forshew at 2, Fig. 1B (depicting the targeted multiplex amplification, wherein "[m]ultiple regions were amplified in parallel. An initial preamplification step was performed for 15 cycles using a pool of the target-specific primer pairs[.]").

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Claim	**Diaz**
	Because *TP53* is a tumor suppressor gene associated with cancer, a POSA would have understood that Sanger sequencing to identify mutations in *TP53* constitutes identification of tumor-specific SNV mutations. Forshew further discloses that "43 single-base substitutions were called (table S3)" and "[t]hese matched 100% of mutations identified by Sanger sequencing and included three additional mutations at low [allele frequencies] that were below detection thresholds of Sanger sequencing (fig. S2)." Forshew at 3. Forshew identifies 40 single-base substitutions (*i.e.*, SNVs) in the ovarian tumor sample. Forshew supplemental materials at 13–14, Table S3. These "single-base substitutions" are tumor-specific SNVs, and a POSA would have understood that such tumor-specific SNVs could be identified in the same manner by performing whole exome sequencing or whole genome sequencing because the whole exome sequences and whole genome sequences include the SNVs.
1(b). evaluating results of the sequencing on the tumor biopsy sample to determine a plurality of target loci specific to the subject, wherein each target locus spans a tumor-specific mutation of the identified plurality of tumor-specific mutations; and	Diaz discloses evaluating results of the sequencing on the tumor biopsy sample to determine a plurality of target loci specific to the subject, wherein each target locus spans a tumor- specific mutation of the identified plurality of tumor-specific mutations. *See, e.g.,* Diaz at 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).

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	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 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: "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. 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. Forshew also discloses evaluating results of the sequencing on the tumor biopsy sample to determine a plurality of target loci specific to the subject, wherein each target locus spans a tumor- specific mutation of the identified plurality of tumor-specific mutations. *See, e.g.,* Forshew at 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,

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	using DNA from 0.08 ml of plasma per amplification, and track[ing] dynamics of all mutations in parallel"); Forshew at 3 ("We therefore applied a two-step amplification process: a limited-cycle preamplification step where all primer sets were used together to capture the starting molecules present in the template, followed by individual amplification to purify and select for intended targets. (Fig. 1B) (Supplementary Methods)."); Forshew supplemental materials at 2 ("Preamplification reactions were carried out in 10-µl reaction volumes containing 50 nM of each forward and reverse target-specific primer . . . Reactions were subjected to 15 cycles of amplification[.]"); Forshew at 2, Fig. 1B (depicting the targeted multiplex amplification, wherein "[m]ultiple regions were amplified in parallel. An initial preamplification step was performed for 15 cycles using a pool of the target-specific primer pairs"); Forshew at 4 ("We applied TAm-Seq to directly identify mutations in plasma of cancer patients."); Forshew at 1 (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 also discloses performing TAm-Seq with a degree of multiplex greater than ten. *See* Forshew Fig 1A; Forshew supplemental materials at 9, Table S1 (disclosing performing TAm-Seq with 48 sequence-specific primer pairs to amplify 5995 bases of genomic sequence). Forshew discloses performing multiplex amplification with a combination of "each forward and reverse target-specific primer" in the same volume. Forshew supplemental materials at 2; *see also id.* at 17-18 (Table S6); Forshew at 6 (Table 2). Kinde also discloses evaluating results of the sequencing on the tumor biopsy sample to determine a plurality of target loci specific to the subject, wherein each target locus spans a tumor- specific mutation of the identified plurality of tumor-specific mutations. *See, e.g.,* Kinde at 3 (describing evaluating tumor biopsy sequencing results); *id.* at 4 ("We designed Safe-SeqS primers (table S4) to detect at least one mutation from each of the 46 patients described in table S3.").; Kinde supplementary table s3.

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1(c). assaying cell-free DNA isolated from a plurality of biological samples obtained from the subject at different time points, wherein the assaying comprises:	Diaz discloses assaying cell-free DNA isolated from a plurality of biological samples obtained from the subject at different time points. *See, e.g,* Diaz at 581-82, Fig. 3, Table 1. As an example, Diaz discloses: "Recent studies have shown that liquid biopsies can be used effectively to monitor the emergence of multiple resistance clones during the course of treatment (Fig 3)." Diaz at 582. As another example, Diaz explains that one of the advantages of ctDNA for monitoring cancer is that "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, "[t]hese 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 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 Fig. 3 illustrates an example of a method comprising assaying the cfDNA DNA isolated from a plurality of biological samples obtained from the subject at different time points to monitor tumor dynamics over time in cfDNA:

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	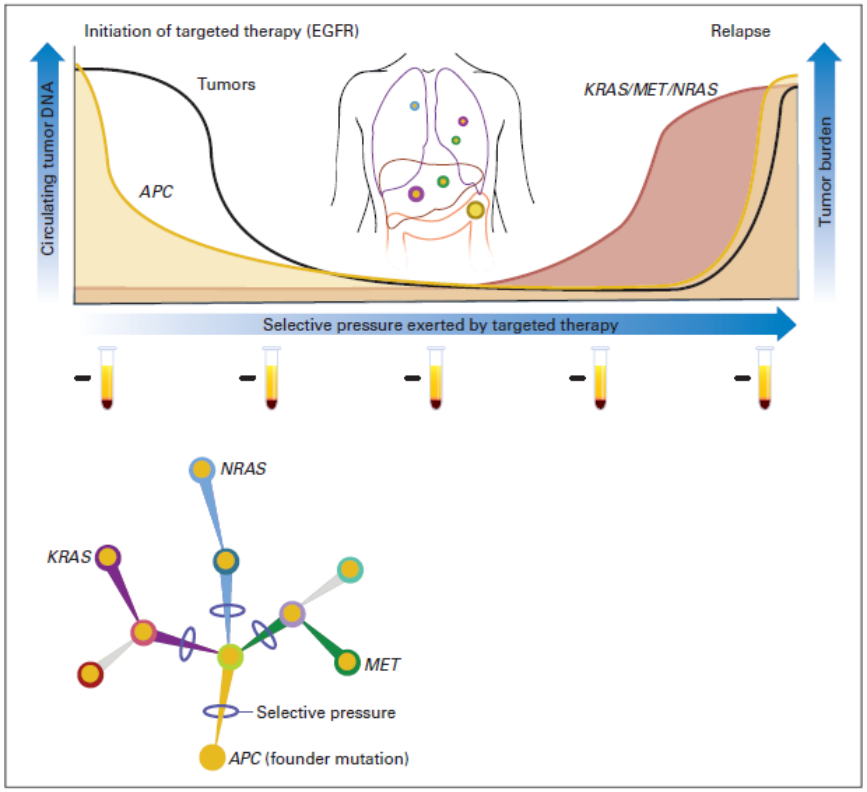 **Fig 3.** Detection of tumor-specific DNA mutations in the blood of patients to monitor response and relapse with targeted therapies. This schematic depicts a representation of a patient with metastatic colorectal cancer. At the time of presentation, DNA from the primary tumor is used to identify the baseline mutation profile; in this case, the tumor is found to be *APC* mutant and *KRAS* wild type (WT). At baseline, evaluation of the patient's plasma DNA only identifies *KRAS* WT fragments. This patient is treated with an anti–epidermal growth factor receptor (EGFR) monoclonal antibody, experiences a clinical response, and has a corresponding decrease in *APC* mutation level, further indicating a decrease in tumor burden. Continuous monitoring of plasma DNA shows the emergence of *KRAS* and *NRAS* mutations and/or *MET* amplification, indicative of the emergence of multiple different resistance clones. Clinical resistance becomes manifest at a later time point. Test tubes represent samples of plasma from which circulating free DNA is extracted and used to monitor the presence of cancer-specific aberrations. As another example, Diaz Table 1 discloses monitoring tumor dynamics as an application of liquid biopsy, which a POSA would understand involves performing liquid biopsies at multiple time points. Forshew also discloses assaying cell-free DNA isolated from a plurality of biological samples obtained from the subject at different time points. *See, e.g.,* Forshew at 2, 4, 7, 8, 9, Figs. 4B-4D, and Supplementary Materials at 19 (Table S7).

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	For example, Forshew discloses: "We studied serial plasma samples collected during follow-up and treatment of two patients with relapsed HGSOC, collected during 104 and 273 days of follow-up and treatment, respectively." Forshew at 8. As another example, Forshew discloses: "Whole-genome sequencing of tumor material was used to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy. Ten mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci . . . Serial plasma samples were collected over the the course of 497 days, both before and after treatment. We performed TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and tracked dynamics of all mutations in parallel. (Fig. 4D)." Forshew at 9, Fig. 4D. As another example, Forshew discloses: "Retrospective analysis by TAm-Seq of FFPE from the primary tumors collected at initial surgery, and three plasma samples collected serially at the time of relapse (5 years and 5 months, 5 years and 7 months, and 6 years after initial surgery), showed that the patient's plasma at relapse contained the TP53 (p.R273H) mutation identified in the ovarian primary tumor." Forshew at 9. As another example, Forshew discloses that "[s]ensitive methods for detecting cancer mutations in plasma" may be useful for "monitoring tumor dynamics over time[.]" Forshew at 1. Forshew further teaches that this "sequencing approach allowed us to monitor changes in tumor burden by sampling only patient plasma over time." Forshew at 2. As another example, Forshew discloses: "DNA extracted from 62 additional plasma samples collected at different time points from 37 patients with advanced HGSOC was amplified in duplicate (table S6), using DNA present in ~0.15 ml of plasma per reaction (range, 0.06 to 0.2 ml)." Forshew at 4. As another example, Forshew Fig. 4B, 4C, and 4D all show results involving assaying cell-free DNA obtained at multiple time points to monitor the frequency of mutant DNA in plasma over time. *See* Forshew at 7 (Fig. 4B, Fig. 4C, Fig. 4D, and accompanying legend).

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	As another example, Forshew discloses: "We selected 10 mutations and designed an amplicon of <120 bp to cover each mutation. We validated these mutations by TAm-Seq in the tumour material and then followed them in serial plasma samples collected from the same patient." Forshew at Table S7.
1(c)[1]. performing targeted multiplex PCR amplification to amplify the plurality of target loci together in the same reaction volume from the isolated cell-free DNA using primers specific to the plurality of target loci for the individual subject; and	Under Natera's apparent interpretation of "the isolated cell-free DNA," Diaz discloses, alone or in combination with the knowledge of a POSA, performing targeted multiplex PCR amplification to amplify the plurality of target loci together in the same reaction volume from the isolated cell-free DNA using primers specific to the plurality of target loci for the individual subject. *See, e.g.,* 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. 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.

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	As another example, Diaz discloses: "In scenarios where only low levels of ctDNA are present (eg, early-stage disease and minimal residual disease), novel genomic methodologies, based on digital PCR, have made such applications possible." Diaz at 584. Under Natera's apparent interpretation of "the isolated cell-free DNA," Forshew also discloses performing targeted multiplex PCR amplification to amplify the plurality of target loci together in the same reaction volume from the isolated cell-free DNA using primers specific to the plurality of target loci for the individual subject. *See, e.g.,* Forshew at 9 (using "[w]hole-genome sequencing of tumor material . . . to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy," wherein "[t]en mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci (table S7)" and performing targeted multiplex amplification that amplifies the ten different tumor-specific SNV mutations in cfDNA from a plasma sample by performing "TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and track[ing] dynamics of all mutations in parallel"); Forshew at 3 ("We therefore applied a two-step amplification process: a limited-cycle preamplification step where all primer sets were used together to capture the starting molecules present in the template, followed by individual amplification to purify and select for intended targets (Fig. 1B) (Supplementary Methods)."); Forshew supplemental materials at 2 ("Preamplification reactions were carried out in 10-µl reaction volumes containing 50 nM of each forward and reverse target-specific primer . . . Reactions were subjected to 15 cycles of amplification[.]"); Forshew at 2, Fig. 1B (depicting the targeted multiplex amplification, wherein "[m]ultiple regions were amplified in parallel. An initial preamplification step was performed for 15 cycles using a pool of the target-specific primer pairs"); Forshew at 4 ("We applied TAm-Seq to directly identify mutations in plasma of cancer patients."); Forshew at 1 (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.")

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	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 1 (Abstract); *see also id.* at 9 ("Serial plasma samples were collected over the course of 497 days, both before and after treatment. We performed TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and tracked dynamics of all mutations in parallel[.]"). Forshew discloses performing multiplex amplification with a combination of "each forward and reverse target-specific primer" in the same volume. Forshew supplemental materials at 2; *see also id.* at 17-18 (Table S6); Forshew at 6 (Table 2). 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 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 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. *Id.* 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

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	same reaction volume from the isolated cell-free DNA using primers specific to the plurality of target loci for the individual subject.
1(c)[2]. performing high-throughput sequencing of the amplified DNA comprising the plurality of target loci to obtain sequence reads,	Diaz discloses performing high-throughput sequencing of the amplified DNA comprising the plurality of target loci to obtain sequence reads. *See, e.g.,* 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). 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 this limitation, Forshew also discloses performing high-throughput sequencing of the amplified DNA comprising the plurality of target loci to obtain sequence reads. *See, e.g.,* Forshew at 2, Fig. 1B; Forshew at 3 (pooling and sequencing DNA that has been amplified through the TAm-Seq method and explaining that "[w]e performed 100-base single-end sequencing (GAIIx sequencer, Illumina), with an additional 10 cycles using the barcode sequencing primer,

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	generating ~30 million reads per lane."); Forshew at 9 ("[W]e demonstrate noninvasive identification of mutant alleles in plasma, at AFs as low as 2%, by targeted deep sequencing of circulating DNA."). To the extent Natera contends that "the amplified DNA" encompasses amplified DNA from an intervening second-round PCR for attachment of sequencing adaptors after the targeted multiplex PCR, 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 Forshew's method without the single-plex amplification step would be an obvious modification. First, Natera has admitted that multiplex PCR amplifications of up to 50 or 100 targets were known in the art. *See, e.g.,* "General Knowledge of a POSA" section in NeoGenomics' Final Invalidity Contentions; U.S. Patent Application Publication 2012/0270212 at [0217] ("the prior art includes disclosures of successful multiplexed PCR reactions involving pools of up to about 50 or 100 primers, and not more."). It would have been obvious to modify Forshew to use any of the other known methods to avoid primer dimer formation during multiplex PCR (e.g., primer design to avoid primer dimers or nested multiplex PCR) instead of an intervening single-plex PCR. *See* "General Knowledge of a POSA" section in NeoGenomics' Final Invalidity Contentions. A POSA would be motivated to use such primer design tools or nested multiplex PCR because such methods allow all amplification steps to be performed in a single tube. *See id.* To the extent Natera contends the claims allow an intervening step to remove spurious amplicons generated in the multiplex amplification before sequencing, such techniques were also known in the art (*e.g.*, automated gel extraction) and would be an obvious modification of Forshew. *Id.* A POSA would be motivated to modify Forshew to use an automated gel extraction method instead of an intervening single-plex PCR because of the advantages of automated gel extraction for removing spurious products

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	such as primer dimers while eliminating variability and cross-contamination, requiring minimal hands-on time, and producing high yield. *See e.g., id.*; Thermo Fisher Scientific Inc. *Pippin™ DNA size selection systems: Improving NGS Data Everywhere* (2013).
1(c)[3]. wherein an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA having the SNV locus is detected from the sequence reads	Diaz discloses that an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA having the SNV locus is detected from the sequence reads. *See, e.g.,* Diaz at 580, Fig. 1. 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 581. 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 581 (citing Kinde 2011). Young discloses one such barcoding strategy to increase overall sensitivity in the context of NGS. *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. 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

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	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%.") 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: Technique / Sensitivity / Optimal Application Sanger sequencing — > 10% — Tumor tissue Pyrosequencing — 10% — Tumor tissue Next-generation seqeuncing — 2% — Tumor tissue Quantative 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 Under Natera's interpretation of the claim, Forshew teaches detection of an SNV because "once you have performed Step 1A and Step 1B and the first part of Step 1C and then you sequence, you will necessarily have identified the SNV if it exists." *See* Metzker deposition 2/11/2025 at 29:1-12. Forshew discloses "[calling] point mutations in a sample only if nonreference AFs were above the respective substitution-specific background distribution at a high confidence margin (0.9995 or

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

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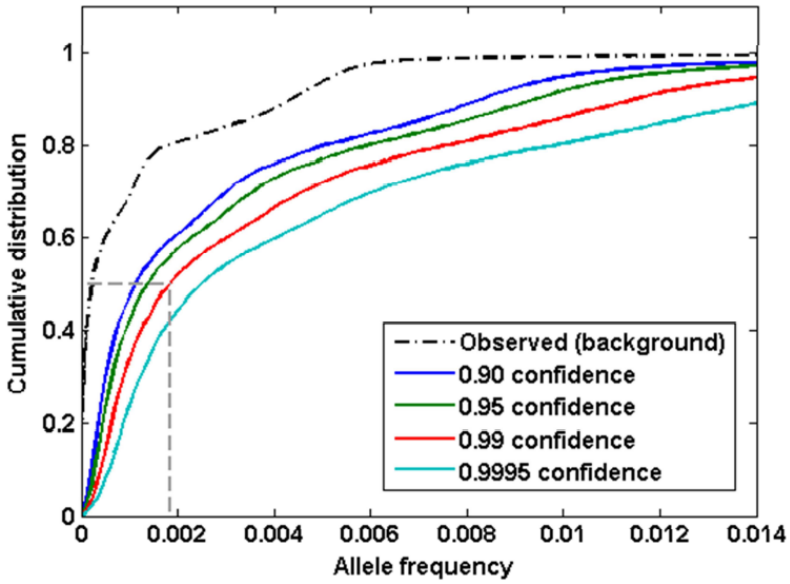
	Supplemental Figure S3 shows some minimal detectable frequencies close to or below 0.015%, even with a confidence margin of 90%. Forshew at Figure S3. A POSA reading Forshew would understand that the minimal detectable frequencies would be much lower without a requirement for 90% confidence. *See* Forshew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

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	is present in less than or equal to 0.015% of the cfDNA because it discloses that background error levels for some substitution types is less than or equal to 0.015%. *See* Forshew Figure S3. As another example, Forshew discloses that "For each locus and base, the distribution of non-reference background allele frequencies/reads was modeled as a Normal distribution and as a Poisson distribution, fitting to a Normal distribution and using the mean number of observed nonreference reads in a set of representative barcodes as the parameter for the Poisson distribution." Forshew Supplementary Materials at 4. As another example, Forshew discloses that "When applying TAm-Seq to measure a pre-defined mutation (as opposed to screening nearly 18,000 possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus. We used confidence margins of ~0.95 using a Normal distribution model for each examined substitution, and requiring a minimum of 10 representative reads, in at least one amplified library per sample. When a mutation was positively detected, its allele frequency was estimated by averaging the allele frequencies in all replicates after subtracting substitution-specific background frequencies." Forshew Supplementary Materials at 4. As another example, Forshew Figure 1C and Figure 1D (reproduced below) show that the background frequency of nonreference alleles was less than 0.015% or below 0.01% for some SNV loci and types of base substitutions (*see* Forshew at 3 (describing Fig. 1C and Fig. 1D)):

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	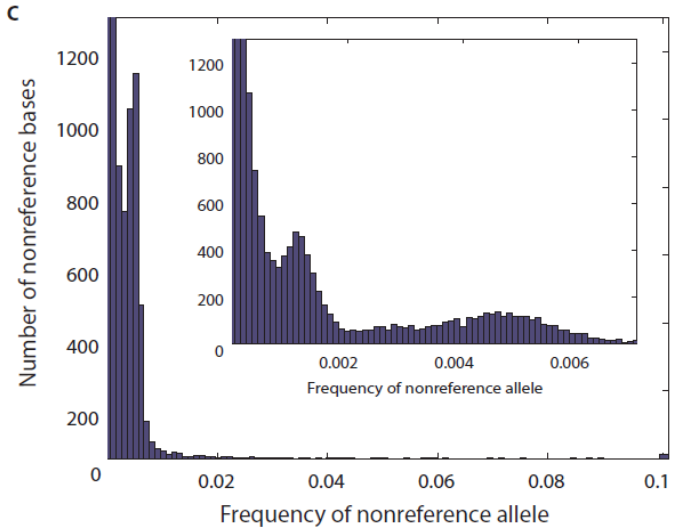 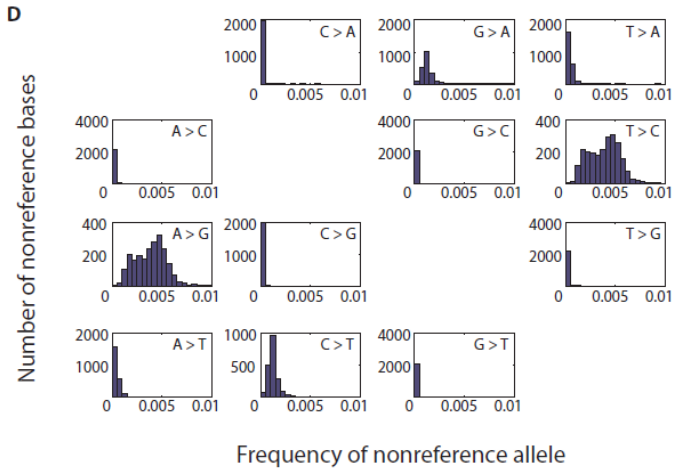

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	As another example:

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	A 1R--> Tp53_reference_NC000017.10plusSNP_F_Synthesis_12387.scf--> 1S--> P53_exon5_JBLAB-104_A4_S1_M13R.ab1--> 1S--> P53_exon5_JBLAB-104_A4_S1_M13R.ab1 **B** 1R<-- Tp53_reference_NC000017.10plusSNP_R_Synthesis_11388.scf<-- 1S<-- P53_exon4_JBLAB-111_S1_M13F.ab1 1S<-- P53_exon4_JBLAB-111_S1_M13F.ab1<--

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	Forshew Supplementary Materials at Figure S2. Forshew teaches that identifying patient-specific mutations comprises sequencing matched tumor and normal tissue samples from a patient. *See* Forshew Supplementary Materials at 19. As to the degree of sensitivity, Forshew specifically teaches the importance of increasing sensitivity and how to improve sensitivity of the TAm-Seq assay, including by increasing sequencing depth and fidelity. Forshew at 10. Further evidence of motivation and reasonable expectation of success is found in NEOGEN00275278–NEOGEN00275334 and NEOGEN00275270–NEOGEN00275277. As an example, NEOGEN00275278 describes experiments performed by Forshew around March 2011 demonstrating successful detection of TP53 mutations in cell-free DNA. For example, NEOGEN00275278 states: "Last week we successfully identified the same mutation by sequencing cell free DNA from the blood directly. To do this we extracted the DNA from blood plasma at two different treatment time points. We then pre amplified the DNA using primers to a number of genes including TP53. We then put this preamplified DNA on a Fluidigm Access Array before sequencing with an Illumina sequencer. We were able to sequence all of TP53 and PTEN and a number of other regions. Through this we identified the same mutation. In the first time point we found that 17% of this DNA was mutated (336/2014 reads). At the second time point this had dropped to 1% (21/2614 reads)." As another example, Forshew discloses: "When applying TAm-Seq to measure a predefined mutation (as opposed to screening thousands of possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus." Forshew at 3. Therefore, Forshew explains, "[f]alse detection is less likely, and criteria for confident mutation detection for a predefined substitution can be less stringent than those described above for de novo mutation identification (Supplementary Methods)." *Id.* As another example, Forshew discloses that "[the] minimal nonreference AFs that could be detected depend on the read depth and background rates of nonreference reads, which vary per locus and substitution type. Minimal detectable frequencies increase when higher confidence margins are used (Supplementary Methods) and had a median value of 0.14% at confidence margin of 0.95 and 0.18% at

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	confidence margin of 0.99 (fig. S3)." Forshew at 3. To the extent Natera contends that the claims do not require a particular confidence threshold for an SNV or a particular clinical sensitivity, a POSA reading Forshew would understand that an SNV that is present at 0.015% could be detected using TAm-Seq with a lower confidence margin. As another example, Forshew discloses: "The minimal detectable frequency would also be limited if a minimal number of reads is applied for confident mutation detection; for example, a minimum of 10 reads implies that sequencing depth of 5000 would be required to detect mutations at AF as low as 0.2%. For alleles present at ~10 or fewer copies in the starting template, reproducibility would also be limited by sampling noise, because these alleles may be over- or underrepresented in any particular reaction." As another example, Forshew discloses that "[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols." 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. 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

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	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 could offer an alternative with comparable sensitivity and specificity that is easy to implement in a clinical sequencing lab." Young at 1611.

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	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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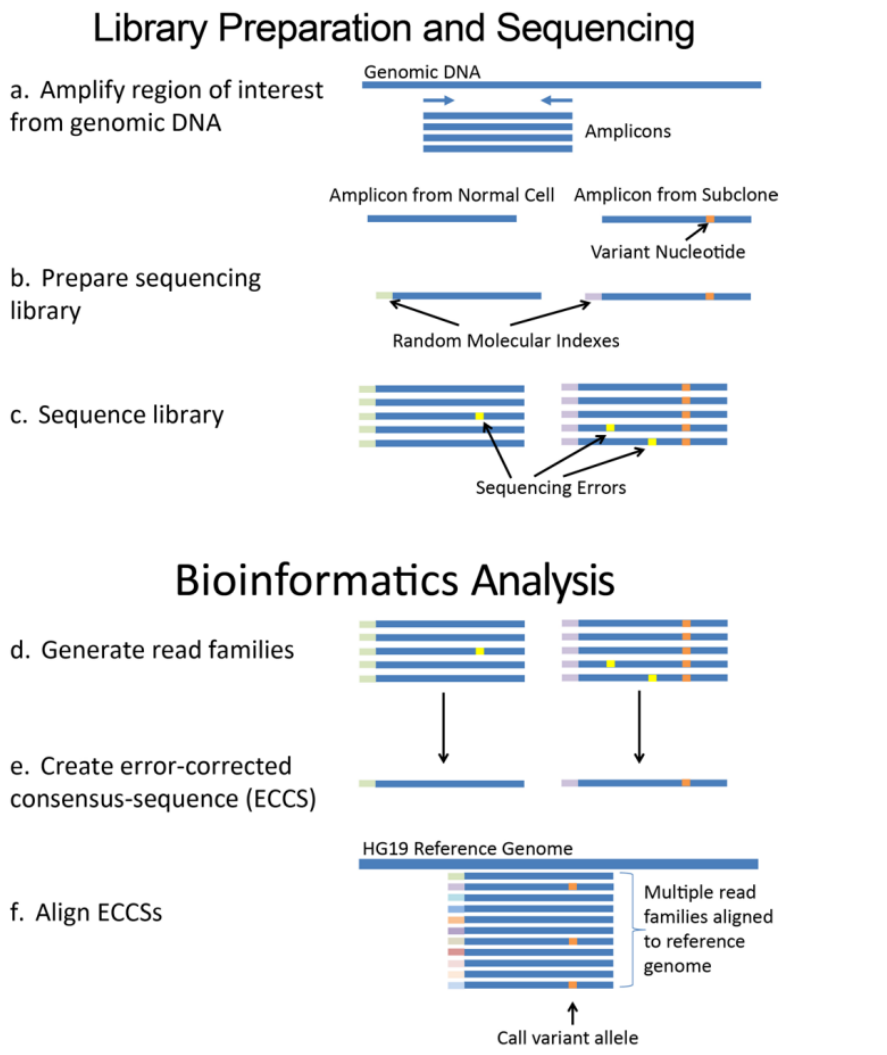
Supplementary Figure 1. Error-corrected sequencing workflow. Schematic depiction of library preparation and bioinformatics analysis for generating read families and error-corrected consensus sequences.



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	Young Supplementary Tables and Figures at Supplementary Figure 1; *see also* Young at 1610. Kinde also discloses a method for detecting an SNV that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus. 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.

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	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 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 ≥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 4, 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

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	mutations to be detected are known *a priori* rather than in an agnostic screening context. Kinde Supplementary Materials at 9. Forshew also renders obvious for a POSA to detect an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus. Obtaining greater sensitivity by increasing sequencing depth was well-known in the art and a POSA would have had a reasonable expectation of achieving a sensitivity of 0.015% because this had already been accomplished before the '596 patent's priority date. *See, e.g.,* Forshew at 3 ("Background frequencies of nonreference reads were ~0.1% (median, 0.03%; mean, 0.2%; in keeping with Q30 quality threshold applied)"); Forshew supplemental materials at 7, Fig. S3 (showing frequencies that could be detected with different confidence levels about the background read rate, including frequencies of 0.015% and below); Forshew at 9 ("Enhanced detection down to 1 mutant allele in 10,000 or more wild-type alleles can be obtained[.]"); Forshew supplemental materials at 17-19, Tables S6—S7 (identification of tumor mutations that are single nucleotide changes or substitutions in a genome, *i.e.,* SNVs) As to the degree of sensitivity, Forshew specifically teaches the importance of increasing sensitivity and how to improve sensitivity of the TAm-Seq assay, including by increasing sequencing depth and fidelity. Forshew at 10. Further evidence of motivation and reasonable expectation of success is found in NEOGEN00275278–NEOGEN00275334 and NEOGEN00275270–NEOGEN0027527. As an example, NEOGEN00275278 describes experiments performed by Forshew around March 2011 demonstrating successful detection of TP53 mutations in cell-free DNA. For example, 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

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	was mutated (336/2014 reads). At the second time point this had dropped to 1% (21/2614 reads)." NEOGEN00275278 at -283. As another example, Forshew discloses: "When applying TAm-Seq to measure a predefined mutation (as opposed to screening thousands of possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus." Forshew at 3. Therefore, Forshew explains, "[f]alse detection is less likely, and criteria for confident mutation detection for a predefined substitution can be less stringent than those described above for de novo mutation identification (Supplementary Methods)." *Id.* As another example, Forshew discloses that "[the] minimal nonreference AFs that could be detected depend on the read depth and background rates of nonreference reads, which vary per locus and substitution type. Minimal detectable frequencies increase when higher confidence margins are used (Supplementary Methods) and had a median value of 0.14% at confidence margin of 0.95 and 0.18% at confidence margin of 0.99 (fig. S3)." Forshew at 3. To the extent Natera contends that the claims do not require a particular confidence threshold for an SNV or a particular clinical sensitivity, a POSA reading Forshew would understand that an SNV that is present at 0.015% could be detected using TAm-Seq with a lower confidence margin. As another example, Forshew discloses: "The minimal detectable frequency would also be limited if a minimal number of reads is applied for confident mutation detection; for example, a minimum of 10 reads implies that sequencing depth of 5000 would be required to detect mutations at AF as low as 0.2%. For alleles present at ~10 or fewer copies in the starting template, reproducibility would also be limited by sampling noise, because these alleles may be over- or underrepresented in any particular reaction." Forshew at 3. As another example, Forshew discloses that "[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols." Forshew at 10.

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	NEOGEN00275326 and NEOGEN00275329 provide further evidence of motivation and reasonable expectation of success for increased read depths. *See, e.g.*, NEOGEN00275326 (describing tables plotting the "number of reads for each barcode according to which block of 48 barcodes it belongs to") and NEOGEN00275329 (providing read counts of greater than 50,000 for some loci (e.g., in columns labeled FLD0200, FLD0233, FLD0234, FLD0235, FLD0236, FLD0237, FLD0238, and FLD0239). As Forshew specifically teaches, "[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols." Forshew at 10. *See also* Forshew at 3 ("The minimal nonreference AFs that could be detected depend on the read depth and background rates of nonreference reads… for example, a minimum of 10 reads implies that sequencing depth of 5000 would be required to detect mutations at AF as low as 0.2%.").
11. The method of claim 1, wherein amplifying comprises targeted multiplex PCR amplification of 10 to 50 of the target loci.	In view of Diaz alone or in combination with the knowledge of a POSA, as well as in combination with the teachings of Forshew and/or Kinde, it would have been obvious to a POSA to perform targeted multiplex PCR amplification of 10 to 50 of the target loci. *See, e.g.,* Diaz at 580, Fig. 1. 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. For example, Forshew teaches performing targeted multiplex PCR amplification of 10 to 50 of the target loci. *See, e.g.,* Forshew at 9 (using "[w]hole-genome sequencing of tumor material . . . to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy," wherein "[t]en mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci (table S7)" and performing targeted multiplex amplification that amplifies the ten different tumor-

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	specific SNV mutations in cfDNA from a plasma sample by performing "TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and track[ing] dynamics of all mutations in parallel"); Forshew at 3 ("We therefore applied a two-step amplification process: a limited-cycle preamplification step where all primer sets were used together to capture the starting molecules present in the template, followed by individual amplification to purify and select for intended targets. (Fig. 1B) (Supplementary Methods)."); Forshew supplemental materials at 2 ("Preamplification reactions were carried out in 10-µl reaction volumes containing 50 nM of each forward and reverse target-specific primer . . . Reactions were subjected to 15 cycles of amplification."); Forshew at 2, Fig. 1B (depicting the targeted multiplex amplification, wherein "[m]ultiple regions were amplified in parallel. An initial preamplification step was performed for 15 cycles using a pool of the target-specific primer pairs"). Forshew further discloses that "[p]lasma of cancer patients contains cell-free tumor DNA that carries information on tumor mutations and tumor burden" and teaches the use of TAm-Seq to monitor cfDNA. Forshew at 1 (Abstract); *see also* Forshew at 9 ("Serial plasma samples were collected over the course of 497 days, both before and after treatment. We performed TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and tracked dynamics of all mutations in parallel."). Forshew also discloses performing TAm-Seq with a degree of multiplex greater than ten. *See* Forshew Fig 1A, Forshew supplemental materials at 9, Table S1 (disclosing performing TAm-Seq with 48 sequence-specific primer pairs to amplify 5995 bases of genomic sequence). Forshew discloses performing multiplex amplification with a combination of "each forward and reverse target-specific primer" in the same volume. Forshew supplemental materials at 2; *see also id.* at 17-18 (Table S6); Forshew at 6 (Table 2). Furthermore, a POSA would have a reasonable expectation of success 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. *See, e.g.,* 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

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	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"). For example, Kinde also discloses performing targeted multiplex PCR amplification of 10 to 50 of the target loci. 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 also 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 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.
12. The method of claim 1, wherein the method further comprises detecting recurrence and/or metastases of the cancer from the tumor- specific	Diaz discloses wherein the method further comprises detecting recurrence and/or metastases of the cancer from the tumor- specific mutations detected in the cell-free DNA. *See, e.g.,* Diaz at 582, 584, Table 1, Fig. 3. 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:

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mutations detected in the cell-free DNA.	**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 As another example, Diaz discloses that "[a]ll patients with detectable postoperative levels of ctDNA experienced recurrence, whereas all patients with undetectable postoperative levels of ctDNA remained disease free." Diaz at 582. 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. Sensitivity will rely on the ability to detect low levels of ctDNA released from micrometastatic deposits not detectable by imaging or other diagnostic modalities." Diaz at 582. As another example, Diaz Fig.3 discloses a method comprising monitoring for metastatic mutations in plasma DNA.

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	As another example, Diaz discloses that "[c]irculating tumor DNA is a potential marker of residual disease after resection and may determine which patients will experience recurrence." Diaz at 582.
18. The method of claim 1, wherein step (a) comprises performing whole exome sequencing on a tumor biopsy sample of the subject to identify the plurality of tumor-specific mutations.	Diaz, in combination with the knowledge of a POSA, as well as in combination with the teachings of Forshew, Bashashati, or Kinde, discloses performing whole exome sequencing on a tumor biopsy sample of the subject to identify the plurality of tumor-specific mutations. *See, e.g.,* Diaz at 580, 582, Fig. 1; *see also* limitation 1(a) above. For example, Diaz discloses methods comprising sequencing a patients tumor to identify "personalized markers based on the unique mutational profiles of resected tumors" and explains that such methods "thus hav[e] exquisitely high specificity." Diaz at 582. As an example, Diaz cites Forshew 2012, which discloses whole genome sequencing to identify the plurality of tumor specific mutations. Diaz at 580, 582, Fig. 1. *See, e.g.,* Forshew at 9 (performing "[w]hole-genome sequencing of tumor material [] to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy."); Forshew supplemental materials at 19, Table S7 (identifying mutations that are single nucleotide changes (*i.e.*, SNVs)); Forshew at 3 (disclosing the use of "TAm-Seq to sequence DNA extracted from 47 formalin-fixed, paraffin-embedded (FFPE) tumor specimens of ovarian cancers (table S3)" and that these samples "were also sequenced for TP53 by Sanger sequencing"). As another example, Forshew discloses: "Whole-genome sequencing of tumor material was used to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy. Ten mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci (table S7). Serial plasma samples were collected over the course of 497 days, both before and after treatment." Forshew at 9.

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

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	Kinde also discloses performing whole exome sequencing on a tumor biopsy sample of the subject to identify the plurality of tumor-specific mutations. *See* Kinde at 3 ("somatic mutations in [] tumors were identified through whole exome sequencing . . . In each of the 46 cases, we identified at least one somatic mutation (table S3) that was confirmed by an independent assay"); *id.* at 5 (describing identifying the tumor-specific mutations in Pap specimens from the corresponding patients). Also, a POSA would reasonably have expected to succeed because the prior art such as Forshew and Kinde taught a POSA how to perform whole genome or whole exome sequencing to tumor-specific mutations. *See, e.g.*, Forshew at 7, Figure 4B; Kinde at 3 ("[s]omatic mutations in [] tumors were identified through whole exome sequencing . . . In each of the 46 cases, we identified at least one somatic mutation (table S3) that was confirmed by an independent assay"); *id.* at 5 (describing identifying the tumor-specific mutations in Pap specimens from the corresponding patients).

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