UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE PATENT TRIAL AND APPEAL BOARD

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NEOGENOMICS LABORATORIES, INC.,
Petitioner,

v.

NATERA, INC.,
Patent Owner

IPR
U.S. Patent No. 11,530,454

EXPERT DECLARATION OF NIALL LENNON, PH.D IN SUPPORT OF PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 11,530,454

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5. Claim 5: The method of claim 1, wherein the SNV mutations comprise one or more clonal SNV mutations and one or more subclonal SNV mutations. 105

6. Claim 6: The method of claim 1, wherein the tumor sample of the subject is a tumor tissue sample. 105

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7. Claim 8: The method of claim 1, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation. 106

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9. Claim 10: The method of claim 1, wherein the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample. 110

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20. Claim 22: The method of claim 14, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation. 130

21. Claim 23: The method of claim 14, wherein the targeted multiplex amplification amplifies 50 to 100 target loci each encompassing a different tumor-specific SNV mutation. 130

22. Claim 24: The method of claim 14, wherein the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample. 130

23. Claim 25: The method of claim 14, wherein the method further comprises performing barcoding PCR prior to the sequencing. 131

24. Claim 26: The method of claim 14, wherein the method further comprises detecting recurrence and/or metastases of the cancer from the SNV mutations detected in the cell-free DNA. 131

25. Claim 27: The method of claim 14, wherein the cancer is colorectal cancer, lung cancer, bladder cancer, or breast cancer. 132

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I. INTRODUCTION

1. My name is Dr. Niall Lennon. I have been retained as an independent expert witness on behalf of NeoGenomics Laboratories, Inc. ("NeoGenomics") in the above-captioned *inter partes* review ("IPR") to provide my independent evaluation and opinions with respect to the validity of all of the claims of U.S. Patent No. 11,530,454 ("the '454 patent").

2. This Declaration sets forth my evaluation and opinions based on my knowledge, skills, education, and experience, and the materials I have considered.

3. In evaluating the materials before me and forming my opinions, I have considered a publicly-available redacted version of the opinion of Dr. Brian Van Ness that was filed in a parallel district court action. EX1006. While I considered Dr. Van Ness' declaration, and there may be similarities in the wording or opinions reached, the analysis and opinions expressed herein are my own.

4. As I explain below, it is my opinion that all of the claims of the '454 patent are invalid under 35 U.S.C. § 103. Claims 1-6, 8-20, and 22-28 of the '454 patent are rendered obvious by Forshew et al., "Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA," Science Translational Medicine, Vol. 4, Issue 136 (May 30, 2012) ("Forshew") (EX1003) in view of the knowledge of a person of ordinary skill in the art ("POSA"). Dependent claims 3-5, 7, 17-19, and 21 of the '454 patent are rendered obvious over

Forshew in view of Bai et al., "Detection and Clinical Significance of Intratumoral *EGFR* Mutational Heterogeneity in Chinese Patients with Advanced Non-Small Cell Lung Cancer," PLOS ONE, Vol. 8, Issue 2 (Feb. 2013) ("Bai") (EX1005) and/or Bashashati et al., "Distinct evolutionary trajectories of primary high-grade serous ovarian cancers revealed through spatial mutational profiling," Journal of Pathology, Vol. 231 (2013) ("Bashashati") (EX1004) and in view of a POSA's knowledge. Claims 1-6, 8-20, and 22-28 of the '454 patent are rendered obvious by Forshew in view of Bentley et al., Patent Application Publication No. 2010/0261189 A1, titled "System and Method for Detection of HLA Viruses" ("Bentley") (EX1057) and/or Narayan et al., "Ultrasensitive Measurement of Hotspot Mutations in Tumor DNA in Blood Using Error-Suppressed Multiplexed Deep Sequencing," Cancer Res. 72(14) (2012) ("Narayan") (EX1086) and in view of a POSA's knowledge.

II. QUALIFICATIONS

5. I am currently part of the Institute Leadership team at the Broad Institute of MIT and Harvard, one of the largest genome sequencing centers in the world. I am also currently the Senior Director of the Genomics Platform, an Institute Scientist, and an Associate Director of the Gerstner Center for Cancer Diagnostics. Specifically, at the Gerstner Center, I evaluate new and upcoming techniques with respect to liquid biopsies. As part of my role, I am responsible for the technology and clinical development strategy for the Genomics Platform at the

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Broad. I am directly responsible for the evaluation, optimization, and application of Next-Generation Sequencing ("NGS") for clinical trials, research, and diagnostics. I also oversee custom method development for use on NGS platforms, such as Illumina's line of sequencing instruments. I am also the founder and current Chairman of the Board of Broad Clinical Laboratories, LLC at the Broad Institute, a CLIA/CAP clinical laboratory that performs cancer and inherited disease molecular assays.

6. Prior to my current position, I was the Director of Technology and Clinical Development at the Broad. Before that I was a Research Scientist for the Genome Sequencing Platform of the Broad. Prior to joining the Broad I was a Postdoctoral Research Fellow at Harvard Medical School. I have over 18 years of experience studying and researching molecular biotechnology, including nucleic acid sequencing and its application to cancer, germline, and viral genomics.

7. I have authored over 80 peer reviewed articles, including articles directed to genomic technologies and the application of sequencing technologies to cancer and viral genomics.

8. I currently serve or have served on technical advisory boards for a number of NGS companies, including Genturi Inc., Swift Biosciences, and FYR Diagnostics. I have been a member of a Key Opinion Leader panel for New England Biolabs. I am an Adjunct Assistant Professor in the Department of

Biomedical Engineering at Tufts University, where I teach Molecular Biotechnology to graduate students.

9. In addition to membership of advisory groups for NGS reagent companies (NEB and Swift) I have extensive experience collaborating with, evaluating, and implementing novel reagent formulations and kits for sample preparation and sequencing. I have worked closely over the years with the R&D teams of several NGS kit and instrumentation companies including Kapa Biosystems (now part of Roche), NEB, Swift Biosciences, Watchmaker Genomics, IDT, Twist Biosciences, Illumina, Ion Torrent, and others. In many cases the purpose of the collaboration is to provide feedback and work with the product development teams who are hoping to bring new methods and kits to the market to support a particular aspect of NGS sample preparation.

10. Exhibit 1007 is a copy of my curriculum vitae.

11. I am being compensated for my time at my standard consulting rate, which is $600 per hour. My compensation does not depend in any way on the outcome of this proceeding or my opinions in this declaration.

III. LEGAL STANDARDS

12. I am not a lawyer. I have been informed by counsel regarding various legal standards, which are described below. I have applied these legal standards in forming my opinions in this declaration.

A. Claim Construction

13. I understand that the words of the claims of a patent are generally given their plain and ordinary meaning from the perspective of a POSA at the time of the claimed invention. I also understand that the claims of a patent are construed in light of the language of the claims, the patent specification, and the prosecution history of the patent (including prior art references that were cited during the prosecution of the patent). I have provided my opinions regarding how certain claim terms of the '454 patent should be construed below. *See infra* Section VIII. In forming my opinions, I have given all other claim terms of the '454 patent their plain and ordinary meaning as would have been understood by a POSA at the time of the claimed invention.

B. Obviousness

14. I understand that a claimed invention is "obvious" if the differences between the claimed subject matter and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a POSA.

15. I understand that a POSA provides a reference point from which the prior art and claimed invention should be viewed. This reference point prevents a POSA from using one's insight or hindsight in deciding whether a claim is obvious.

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16. I understand that the obviousness analysis requires a comparison of the claim language, including as construed by the Court, to the prior art on a limitation-by-limitation basis.

17. I understand that in assessing whether a claimed invention would have been obvious at the time of the purported invention to a POSA, the following factors are considered: (1) the scope and content of the prior art; (2) the differences between the art and the claims at issue; (3) the level of ordinary skill in the art; and (4) the existence of secondary indicia of non-obviousness or obviousness.

18. I understand that a claim can be obvious in light of a single reference, without the need to combine multiple prior art references, if the elements of the claim that are not found explicitly or inherently in the reference can be supplied by the knowledge or common sense of one of skill in the art.

19. I understand that an obviousness evaluation can be based on a combination of multiple prior art references. I understand that such a combination must meet certain requirements.

20. I understand the prior art must teach or suggest all the requirements of the invention as recited in the claims. I understand the prior art, coupled with the knowledge generally available in the art at the time of the purported invention, must contain some suggestion or incentive that would have motivated a POSA to modify a reference or to combine references. I also understand the proposed modification

of the prior art must have had a reasonable expectation of success, determined from the vantage point of a POSA at the time the purported invention was made.

21. I understand that the prior art references themselves may provide a suggestion, motivation, or reason to combine, but other times the nexus linking two or more prior art references is simply common sense. I further understand that an obviousness analysis recognizes that market demand, rather than scientific literature, often drives innovation, and that a motivation to combine references may be supplied by the direction of the marketplace.

22. I understand that if a technique has been used to improve one device, and a POSA would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond the person's skill.

23. I understand that practical and common sense considerations should guide a proper obviousness analysis, because familiar items may have obvious uses beyond their primary purposes. I further understand that a POSA looking to overcome a problem will often be able to fit the teachings of multiple publications together like pieces of a puzzle, although the prior art need not be like two puzzle pieces that must fit perfectly together.

24. I understand that obviousness analysis therefore takes into account the inferences and creative steps that a POSA would employ under the circumstances.

25. I understand that a particular combination may be proven obvious by showing that it was obvious to try the combination. For example, when there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a POSA has good reason to pursue the known options within the person's technical grasp because the result is likely the product not of innovation but of ordinary skill and common sense.

26. I understand that the combination of familiar elements according to known methods may be proven obvious when it does no more than yield predictable results. When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a POSA can implement a predictable variation, then that obviousness likely bars its patentability.

27. I understand that a proper obviousness analysis focuses on what was known or obvious to a POSA, not just the patentee. Accordingly, I understand that any need or problem known in the field of endeavor at the time of invention and addressed by the patent can provide a reason for combining the elements in the manner claimed.

28. I understand that a POSA could have combined two pieces of prior art or substituted one prior art element for another if the substitution can be made with predictable results, even if the swapped-in element is different from the swapped-

out element. In other words, the relevant question is whether prior art techniques are interoperable with respect to one another, such that that a POSA would view them as a design choice, or whether a POSA could apply prior art techniques into a new combined method or system.

29. In sum, my understanding is that prior art teachings are properly combined where a POSA having the understanding and knowledge reflected in the prior art and motivated by the general problem facing the inventor would have been led to make the combination of elements recited in the claims. Under this analysis, the prior art references themselves, or any need or problem known in the field of endeavor at the time of the purported invention, can provide a reason for combining the elements of multiple prior art references in the claimed manner.

30. I understand that obviousness must be shown by a preponderance of the evidence.

31. I understand that certain objective factors, sometimes known as objective indicia must be taken into account in determining whether a claimed invention would have been obvious. I understand that the following is a nonexclusive list of secondary considerations to analyze for this inquiry: (i) a strong showing of commercial success of a product covered by the patent claims that can be attributed to the merits of the invention; (ii) a long felt but unmet need in the prior art that was satisfied by the invention of the patent; (iii) the willingness of industry

members to license the patent at issue; (iv) evidence that those of skill in the art were skeptical as to the merits of the invention, or that prior art references taught away from the invention; (v) evidence that those of skill in the art were surprised by the capabilities of the claimed invention; (vi) praise of the invention by others skilled in the art; and (vii) deliberate copying of the invention. I further understand that contemporaneous and independent invention by others is a secondary consideration supporting an obviousness determination.

32. I understand that any assertion of the above indications must be accompanied by a nexus between the merits of the purported invention and the evidence offered. If there is no such connection between the claimed technology and the secondary evidence, then that evidence does not actually establish the purported invention was nonobvious.

C. Written Description and Priority

33. I understand that the claimed invention(s) in a patent must be supported by written description in any application to which it is entitled to the benefit of priority.

34. I understand that the written description in the specification of the application must describe the claimed invention sufficiently to convey to a POSA that the patentee had possession of the claimed invention at the time of the application. I also understand the specification must describe the claimed

invention sufficiently to convey to a POSA that the applicant actually invented the claimed invention. I also understand that to show possession, the specification must convey the invention to a POSA in such a way that the POSA understands that the invention is possessed by the inventor, and not merely laying out a research plan for a POSA to follow. In other words, I understand that describing a desired outcome without describing how to achieve it is not sufficient.

35. I understand that invalidity for lack of adequate written description must be shown by clear and convincing evidence.

IV. THE '454 PATENT

36. The '454 patent, entitled "Detecting mutations and ploidy in chromosomal segments," issued on December 20, 2022. EX1001.

A. Claims

37. All of the claims of the '454 patent are being challenged in the Petition, including independent claims 1 and 14 and dependent claims 2-13 and 15-28. The claims of the '454 patent recite steps for performing whole exome sequencing or whole genome sequencing on tumor sample DNA from a cancer patient to identify tumor-specific single nucleotide variant ("SNV") mutations, obtaining a blood sample from the subject and amplifying cell-free DNA ("cfDNA") that harbors the identified SNV mutations, sequencing, and analysis to detect the tumor-specific SNV mutations in the cfDNA.

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38. Independent claims 1 and 14 are reproduced below:

1. A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample, the method comprising:

   (a) performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;

   (b) performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume; and

   (c) sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads, wherein the sequencing has a depth of read of at least 50,000 per target locus.

14. A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample, the method comprising:

(a) performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;

(b) performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons, wherein the target loci are amplified together in the same reaction volume; and

(c) sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads, wherein the method is capable of detecting an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus.

B. Priority Date

39. I understand that patents may claim the benefit of, or priority to, prior application(s). The '454 patent includes, in "Related U.S. Application Data," a listing of earlier-filed patent applications. EX1001 at codes (60), (63). Patent Owner provided a Court-ordered interrogatory response as to the priority date for the '454 patent of "by April 21, 2015," the filing date of Application No. 14/692,703 ("the '703 application"). EX1008.010 ("The conception and reduction to practice date of each of the asserted claims is by April 21, 2015."); EX1009.002 ("Natera

shall respond comprehensively to [Interrogatories Nos. 1 and 5] by September 25, 2023."); EX1001 at code (63), claims 1 and 14.

40. The '703 application was the first application in the '454 patent's priority chain with any written description support for whole exome sequencing or whole genome sequencing, as required by the independent claims of the '454 patent. Specifically, this support appears in Example 13, first included in the '703 application. EX1001 at 167:66-168:19; EX1010 ¶ 608.

41. Provisional applications to which the '703 application claims the benefit of priority refer to "long read sequencing" and "phasing," and I am informed that Patent Owner may contend such disclosures constitute written description support for whole exome or whole genome sequencing. *See e.g.*, EX1011 ¶ 98; EX1012 ¶ 97; EX1013.044; EX1014 ¶¶ 107, 372; EX1015 ¶¶ 107, 372; EX1016 ¶ 107; EX1017 ¶¶ 107, 394. Having considered the disclosures in the provisional applications, and the plain meaning of these terms to a POSA, however, neither recitation of "long read sequencing" nor "phasing" constitutes a description or disclosure of either whole exome or whole genome sequencing because a POSA would have understood that both could have been, and often were, accomplished on much smaller portions of genomic material than whole exomes or whole genomes. *See, e.g.*, EX1014 ¶ 35 ("In some embodiments, the phased genetic data for the individual is obtained ***by phasing a portion*** or all ***of region [sic]*** corresponding to a

deletion or duplication in a sample from the individual.") (emphasis added), ¶ 372 ("In some embodiments, ***long read sequencing*** (such as sequencing using the Moleculo Technology developed by Illumina to sequence up to ~ 10 kb) or paired end sequencing ***is used to sequence part*** or all ***of a recombination hotspot***.") (emphases added).

42. For the purposes of this declaration only, I assume April 21, 2015, the filing date of the '703 application, is the priority date of the '454 patent.

C. Prosecution History

43. On May 6, 2022, Patent Owner submitted U.S. Patent Application No. 17/738,354 ("the '354 Application"). EX1018 (5/6/2022 Track One Request). The '354 Application included 15 claims directed to methods for amplifying and sequencing DNA. EX1019 (5/06/2022 Submitted Claims).

44. On July 7, 2022, the Examiner issued a Non-Final Rejection, rejecting all of the then-pending claims except claim 2 as obvious under 35 U.S.C. § 103 over Forshew in view of other prior art references. EX1020.005-.007 (7/7/2022 Non-Final Rejection). Then-pending claim 2 was not rejected as obvious; the claim recited "wherein the high-throughput sequencing has a depth of read of at least 50,000 per target locus." EX1019.001; EX1020.005-007.

NEOGENOMICS EX.1002.021

45. On October 7, 2022, in response to the Examiner's Non-Final Rejection, Patent Owner amended claims 1, 11, 12, and 15, cancelled claim 2, and added claims 16-29. EX1021. Patent Owner asserted:

> Claim 2 stands free of the subject rejections. For the sole purpose of expediting prosecution and without acquiescing to the rejection, Applicant has amended independent claim 1 to incorporate the limitation of previous claim 2, thereby rendering the rejections moot. None of the cited references teaches or suggests sequencing each of the target loci with a depth of read of at least 50,000 per target locus. For at least these reasons, Applicant respectfully request withdrawal of the subject rejections against claims 1 and 3-14.

EX1022.003 (10/7/2022 Remarks Made In Amendment).

46. Moreover, Patent Owner asserted that Forshew did not rely on multiplex amplification, instead relying on single-plex amplification. EX1022.004. Specifically, Patent Owner asserted:

> Furthermore, Forshew's TAm-Seq process was rationally and deliberately designed to rely on single-plex, "*individual amplification to purify and select for intended targets*" (pg. 3). In fact, Forshew had considered but dismissed multiplex targeted amplification – "*Multiplex amplification using a large set of primers could result in nonspecific amplification products and biased coverage*" (Fig. 1B, pg. 3). Forshew deliberately resorted to parallel single-plex amplification on Access Array, which physically separate individual primer pairs and individual reaction volumes to avoid issues such as nonspecific amplification and primer dimer formation. There was no reason or motivation for one of ordinary skill in the art to alter Forshew's TAmSeq process by substituting a step rationally and deliberately designed by Forshew for purpose of addressing the issue of nonspecific amplification, for a step already considered but dismissed by Forshew.

Id. (emphasis in original).

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47. On November 30, 2022, the '454 patent issued. EX1023 (11/30/2022 Issue Notification). In the Notice of Allowance, the Examiner withdrew the obviousness rejections in view of Patent Owner's amendment and arguments. EX1024.007 (11/8/2022 Notice of Allowance).

V. PERSON OF ORDINARY SKILL IN THE ART

48. I understand that claims of a patent are construed from the perspective of a POSA at the time of the claimed invention. I understand that whether claims of a patent are obvious under 35 U.S.C. § 103 is also analyzed from the perspective of a POSA at the time of the claimed invention.

49. In my opinion, a POSA would have had at least (1) a Master's Degree and/or Ph.D. in biochemistry, molecular genetics, pharmacology, or an equivalent discipline with applications in genomics and (2) at least five years of academic or industrial laboratory experience.

50. In my opinion, a POSA would have a deep understanding of genomics, including nucleic acid detection, quantification, and genotyping and would understand the underlying scientific principles to such.

51. In my opinion, a POSA would stay apprised of recent literature on genomic assays, such as multiplex PCR, next-generation nucleic acid sequencing techniques, and the use of cfDNA in genotyping. A POSA would also generally

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understand the processes behind the development of DNA mutation-based illnesses, such as cancers, and would stay apprised of the recent literature in those fields.

52. It is my opinion, as set forth herein, that all of the claims of the '454 patent are obvious under this definition of a POSA, or any other reasonable definition of a POSA.

53. I have been informed that Patent Owner, in a federal district court case in North Carolina, relies on testimony from Dr. Michael Metzker setting forth that a POSA would have had:

> [i] at least a bachelor's degree (with several additional years of laboratory experience) in a life or physical sciences discipline with applications in genomics[;]
> [ii]a thorough understanding of nucleic acid detection, quantification, and genotyping [obtained through further education or graduate or postdoctoral experience;]
> …
>
> [iii] [an][underst[anding] [of] the basic underlying scientific principles of nucleic acid assay, detection, and genotyping techniques[;] [and]
> …
>
> [iv] general knowledge of, and…[ability] to read literature on, nucleic acid assay methods including, for example, amplification by PCR, nucleic acid sequencing including by multiplex or high-throughput sequencing, genotyping, and the detection and quantification of nucleic acids, including cell-free DNA, using techniques such as polymorphism-based genotyping.

NEOGENOMICS EX.1002.024

EX1025 ¶ 32. Patent Owner further contends that "in determining the level of ordinary skill in the art, more education could compensate for less experience, and vice versa." *Id.*

54. To the extent Patent Owner advances Dr. Metzker's definition of POSA, or another alternative definition, in this petition or in the federal court litigation, my opinion regarding obviousness would remain unchanged.

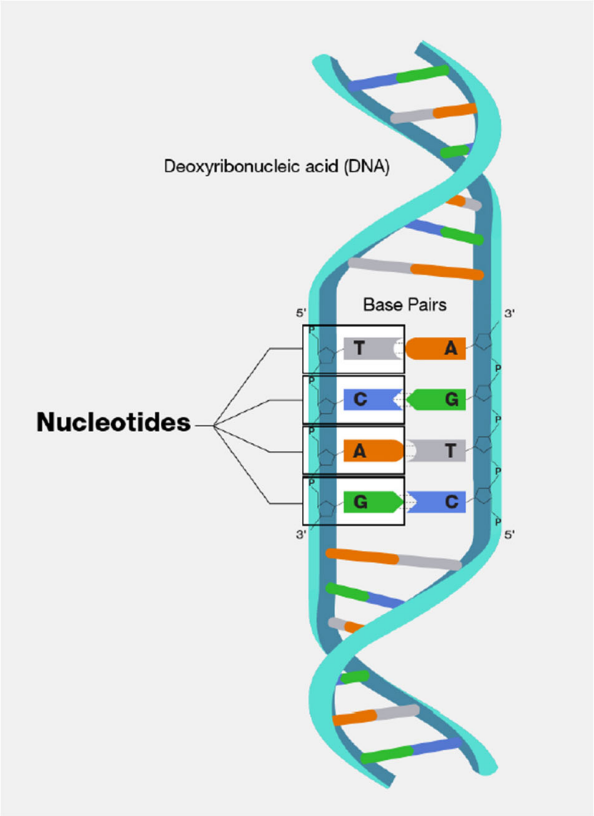
VI. TECHNOLOGY BACKGROUND

A. Cell-Free DNA

55. DNA is a double-stranded polymer comprised of nucleotides. Each nucleotide comprises (1) a deoxyribose sugar molecule, (2) one or more phosphate groups, and (3) a nitrogenous base. There are four types of bases that make up DNA: adenine ("A"), thymine ("T"), cytosine ("C"), and guanine ("G"). Each base has a complementary base – A is complementary to T, while C is complementary to G. EX1026.001; EX1027.001.

56. The two strands of DNA are complementary, connected via complementary base pairing. For example, a single strand of DNA with a sequence of GACT would bind to a complementary strand of DNA with a sequence of CTGA, as shown below:

NEOGENOMICS EX.1002.025



EX1026.002; *see also* EX1027.001.

57. Each strand of DNA has a 5' end and a 3' end. DNA synthesis occurs in the 5' to 3' direction. EX1027.002. The complementary nature of DNA has great significance in biological techniques such as polymerase chain reaction ("PCR") and DNA sequencing that rely on the binding of complementary strands together, a process called hybridization, and the synthesis of a second strand of DNA complementary to a first strand that acts as a template for the synthesis. *See id.* ("The annealing of complementary nucleotide sequences, a process called

hybridization, forms the basis of several DNA technologies that helped launch the biotechnology industry and modern genomics.").

58. In humans, most DNA is generally found within the nucleus of cells, specifically, in large, complex structures called chromosomes. *Id.* at .001; EX1028.001. The nucleus contains all of the material required to maintain, replicate, and use DNA. EX1028.001. DNA is also found outside of cells, and has been linked to apoptosis (programmed cell death), as well as signaling between cells. EX1029.002; EX1030.003. This DNA found outside cells is DNA digested or fragmented by natural processes and then released into the tissue environment, eventually finding its way into the blood. EX1029.002; EX1037.002; EX1126.004.

59. DNA located outside of the cells is referred to as cfDNA and is derived from bodily fluids that are cell-free, such as blood plasma. EX1003.002. cfDNA has been known since 1947 and its presence has been correlated to illnesses, including cancer since the 1970s. EX1030.002. cfDNA in blood plasma may originate from both normal cells and cancer cells and is "detectable in healthy volunteers, patients without cancer, patients with benign tumors, and cancer patients." EX1031.002; *see also id.* ("Circulating tumor DNA (ctDNA) is the portion of circulating DNA specifically derived from cancer cells."); *see also* EX1032.002 ("Circulating DNA fragments carrying tumor-specific sequence

alterations (circulating tumor DNA) are found in the cell-free fraction of blood."); *see also* EX1033 ¶ 3 ("Tumor cells can often be found in the circulation of individuals with advanced cancers."); *see also* EX1030.002 ("The cellular origin of the extracellular DNA was not determined until the 1980s, when Stroun and Anker showed that circulating free DNA was derived from tumor cells. They showed that certain characteristics of tumor DNA were also present in circulating DNA. Further work in the 1990s showed that oncogene mutations, loss of heterozygosity (LOH), and microsatellite shifts can be found in the plasma and serum matching those occurring in primary tumors.").

60. Cancers may be diagnosed and monitored by detecting and analyzing the presence of ctDNA, as well as any changes in the ctDNA. *See* EX1034 at 1:19-28; EX1033 at Abstract ("We apply a highly sensitive approach to quantify circulating tumor DNA (ctDNA) in body samples of patients."); *id.* ¶ 3 ("It has been shown that tumor-derived mutant DNA can also be detected in the cell-free fraction of the blood of people with cancer."); *see also* EX1035 at Abstract ("[M]ethods of detecting and monitoring extracellular mutant oncogenes or tumor-associated DNA found in the plasma or serum fraction of blood"); *see also* EX1036.003 ("One of the most immediate applications of ctDNA has been termed the "liquid biopsy" (20)....once a targeted therapy is initiated in a patient with multiple metastases, clinicians frequently search for early evidence of recurrence or mechanisms

underlying resistance, a scenario in which liquid biopsies are particularly valuable. For example, they can provide temporal measurements of the total tumor burden as well as identify specific mutations that arise during therapy (16, 20, 21, 23, 28, 52).").

61. cfDNA may be analyzed to determine: 1) whether tumor DNA is present, thus showing a recurrence or new tumor development, 2) how much tumor DNA is present, which can indicate the cancer's severity or reaction to treatment, and 3) the differences within the cfDNA collected, which further indicates tumor progression. EX1037.002-.004; EX1036.002-.003.

B. Single Nucleotide Variants (SNVs) and Single Nucleotide Polymorphisms (SNPs)

62. DNA variants can occur naturally (differences between people) or through mutations to an individual's DNA. Cancer is associated with mutations in DNA. These mutations can affect large or small portions of DNA. EX1038.001-.003 (discussing deletions of spans of DNA associated with lung cancer); EX1032.005-.006 (discussing single nucleotide mutations and structural variants, such as deletions of nucleotide spans). Mutations that affect a single nucleotide are called single nucleotide variants ("SNVs") or single nucleotide polymorphisms ("SNPs"). SNVs and SNPs are both single nucleotide variations in DNA. EX1039 at Abstract; EX1032.005-.006. For example, if the DNA of a healthy

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individual includes a TAG**T**GC nucleotide sequence, then the DNA of an individual diagnosed with cancer may instead include a TAG**C**GC sequence (*i.e.*, C instead of T at the fourth nucleotide position).

63. Somatic SNVs are generally defined as single nucleotide mutations that differ from the individual's germ-line (original) DNA, and are often identified in cancerous DNA. EX1039 at Abstract ("Somatic (cancer-only) single nucleotide variants (SNVs) are the simplest class of mutation, yet their identification in DNA sequencing data is confounded by germline polymorphisms, tumour heterogeneity and sequencing and analysis errors."). The '454 patent uses SNV consistently with how the term is used in the art, where SNV is used to describe the detection of cancerous tumor-specific mutations. *See, e.g.*, EX1001 at 41:65-42:3, 171:29-43 (claim 1).

64. The '454 patent defines SNP as "a single nucleotide that may differ between the genomes of two members of the same species. The usage of the term should not imply any limit on the frequency with which each variant occurs." EX1001 at 28:13-17. Single nucleotide changes in a person's germline DNA are typically referred to as SNPs in the art, including in 2015 and before. EX1040 at Abstract, .005 (discussing "detecting genetic variation" and SNPs); EX1111 at Abstract (discussing SNPs in human height variation); *see also id.* ("Single nucleotide polymorphisms (SNPs)...account for only a small fraction of the genetic

variation of complex traits in human populations....We estimated the proportion of variance for human height explained by 294,831 SNPs genotyped on 3,925 unrelated individuals....We show that 45% of variance can be explained by considering all SNPs simultaneously.").

C. Multiplex PCR Amplification

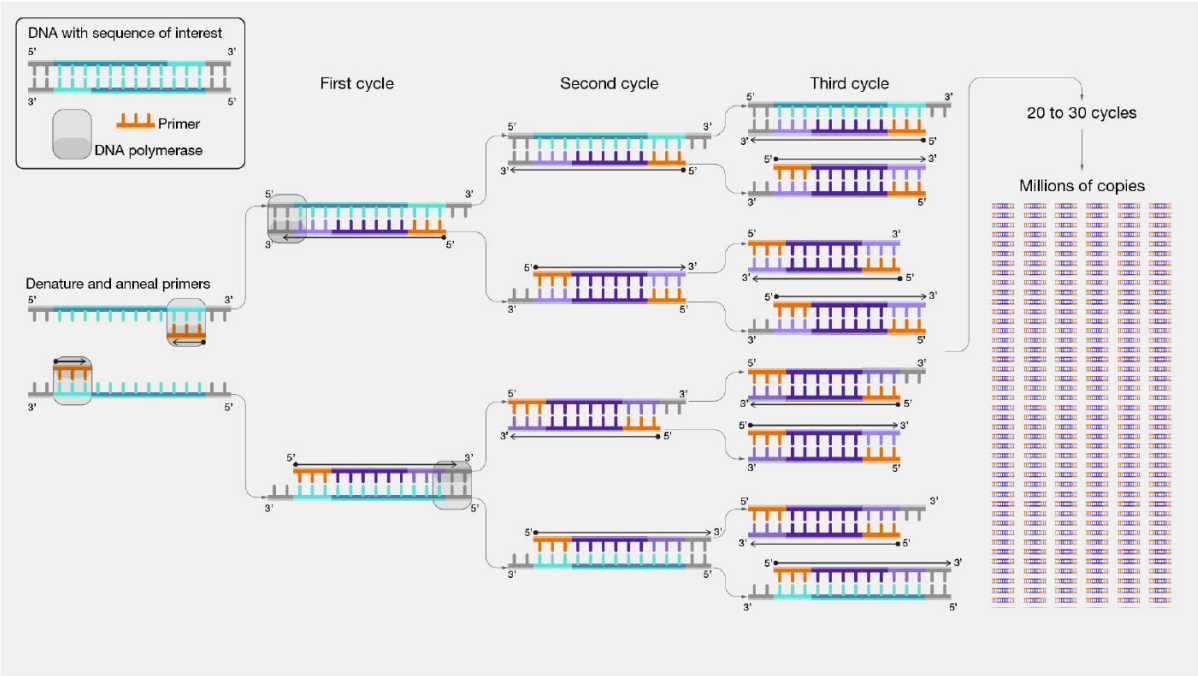
65. A minimum amount of DNA is often required to perform different biotechnology applications or processes, such as DNA sequencing. A sample with a limited amount of DNA must, accordingly, sometimes be amplified or copied prior to performing DNA sequencing with certain sequencing platforms. Amplification is the process of creating multiple copies of DNA. EX1001 at 34:52-53. PCR is one method of amplification, wherein DNA fragments are amplified or copied in cycles using an enzyme called DNA polymerase. The '454 patent discusses using various PCR methods to amplify DNA. *See, e.g.*, EX1001 at 97:52-98:27.

66. As described in further detail below, a typical PCR cycle requires three steps: 1) denaturing the double-stranded DNA to form single-stranded DNA, 2) annealing primers to the resulting single-stranded DNA molecules, and 3) primer extension using the single-stranded DNA molecules as templates for the complementary strands formed. EX1041.002.

67. First, denaturation is the separation of the double-stranded DNA by using heat or through other chemical or physical means. *Id.*

68. Second, annealing primers is the process of binding primers through base pairing to the complementary single-stranded DNA template strands that result from denaturation. Primers can be designed to bind to almost any sequence of DNA. Generally, PCR uses pairs of primers where each individual primer in a pair binds to a complementary sequence at the 3' end of each of the complementary single-stranded DNA molecules, and where each of the single-stranded DNA molecules of the double-stranded DNA can then act as a template for extension of the primer in the 5' to 3' direction to form a further complementary DNA strand. *Id.*

69. Third, primer extension is the process wherein the annealed primers are extended in the 5' to 3' direction by an enzyme, typically a form of DNA polymerase. In extending the primers, the DNA polymerase synthesizes new strands of DNA complementary to the single-stranded template strands, forming double-stranded DNA molecules. *Id.* In extending the primers in the 5' to 3' direction, DNA polymerase synthesizes the new strands by the addition of nucleotides to the 3' end of the annealed primer. This process may be repeated in multiple PCR cycles, as shown below:



EX1092.001.

70. PCR was well-established and well-known in the art prior to 2015. *See, e.g.*, EX1085 at 30:20-31:17; EX1041.

71. Multiplex PCR involves the use of multiple primer pairs to amplify many different target DNA locations ("loci") in a single reaction or container, rather than using a single primer pair to amplify a single target sequence. *See* EX1058 at Abstract ("The present invention provides a method for multiplex polymerase chain reaction (mpxPCR), the simultaneous amplification of different target nucleic acid sequences in a single PCR reaction."); *id.* ¶ 30. Multiplex PCR is faster, less expensive, and less cumbersome compared to performing PCR for each target DNA sequence in a separate reaction or container. EX1079 at Abstract. Multiplex PCR

also reduces the likelihood of missing rare molecules because it avoids the need to partition the sample into individual reactions, each of which will start with less genetic material. EX1003.004 ("Performing single-plex amplification with each of these primer pairs would require dispersing the initial sample into many separate reactions, considerably increasing the probability of sampling errors and allelic loss.").

72. Multiplex PCR has been well-known since long before 2015. For example, the '454 patent incorporates by reference U.S. Patent Application No. 13/300,235 ("the '235 application"). EX1001 at 97:13-20. The '235 application, which was published in 2012, discloses multiplexing greater than "500 loci simultaneously." EX1050 at ¶ 267 ("Whereas traditional multiplex PCRs evaluate up to fifty loci simultaneously, the approach described herein may be used to enable simultaneous evaluation of more than...500 loci simultaneously..."); *see also id.* ¶ 303 ("Prior art demonstrates that ordinary people skilled in the art typically design 100+ bp amplicons...In an embodiment, the amplicons may be designed to be less than 50 bp."); *id.* at ¶¶ 515-16 (Experiment 4 using 800-plex amplification of DNA).

73. As another example, a patent related to the '454 patent, and claiming an earlier priority date, describes that 100 loci could be multiplexed in a single reaction. EX1049 at 85:14-16 ("Methods described in the prior art used to multiplex more than 50 or 100 [loci] in one reaction volume followed by

sequencing…."); *id.* at 48:25-29 ("Also note that the general belief in the art is that multiplexing PCR for sequencing is limited to about 100 assays in the same well. Fluidigm and Rain Dance offer platforms to perform 48 or 1000s of PCR assays in parallel reactions for one sample."); *id.* at 210:35-211:43 (Experiment 4 using 800-plex amplification of DNA).

74. The prior art more generally establishes that multiplex PCR was well-known in the art. For example, Kaper et al., "Parallel Preparation of Targeted Resequencing Libraries from 480 Genomic Regions Using Multiplex PCR on the Access Array™ System," a poster presented at the American Association for Cancer Research (AACR) 101st Annual Meeting in 2010, teaches the use of an "Access Array™" system for scaling up PCR capabilities that comes with 48 sample inputs with 48 primer sets and, thus, performs multiplex PCR. EX1071; EX1072. Kaper further teaches the multiplex amplification of 480 cancer gene exons, where 48 primer pools of 10 primer pairs are used for the reactions, and where the PCR products are then sequenced. EX1072 at Fig. 2.

75. Multiplex PCR reactions involving multiplexing thousands of DNA locations were also well-known in the art. EX1060 ¶ 214 ("multiplex amplifications permit the simultaneous amplification of…as many as tens, hundreds, thousands, or even more polynucleotide sequences of interest."); EX1083 ¶ 119 ("The pre-preamplification can be carried out in multplex [sic]. For example,

target-specific primers for 9216 different target nucleic acids can be employed in one mixture."); *id.* ¶ 175 ("these methods are applicable to identifying the presence of particular polymorphisms (such as SNPs), alleles, or haplotypes, or chromosomal abnormalities, such as amplifications, deletions, or aneuploidy"); EX1107.004-.005 (describing "nested patch PCR" capable of amplifying 90 gene fragments in the same reaction and useful for amplifying 100-1000 DNA fragments); EX1058 at Abstract, ¶ 57 ("The present invention provides a method for multiplex polymerase chain reaction (mpxPCR), the simultaneous amplification of different target nucleic acid sequences in a single PCR reaction" preferably with "at least 100 targets."); EX1109.001 ("A high-throughput genotyping system for scoring single nucleotide polymorphisms (SNPs) has been developed. With this system, >1000 SNPs can be analyzed in a single assay.").

76. By 2005, these kinds of multiplex PCR reactions were commercialized by several companies:

> Very recently, the universal tail concept was applied by a few newly developed systems (Yeakley et al. 2002; Hardenbol et al. 2003; Kennedy et al. 2003; Fan et al. 2004; Matsuzaki et al. 2004). With these systems, universal sequences are attached to the amplicons with different approaches before PCR, allowing >1000 SNP-containing sequences to be amplified in a single reaction. This advance has significantly facilitated high-throughput genotyping. The technique initially described by Yeakley et al. (2002) has been commercialized by Illumina and used in ~65% of the HapMap project (http://www.hapmap.org). The technologies described by Hardenbol et al. (2003) and Kennedy et al. (2003) have also been commercialized

> and used in largescale genetic analyses (Butcher et al. 2004; Zhou et al. 2004).

EX1109.002.

77. By 2015, various DNA polymerases were also commercially available for use in PCR reactions, including polymerases that were able to amplify DNA fragments with high fidelity (few errors). For example, the Q5 DNA Polymerase was commercially available from New England Biolabs and was stated to be "an ideal choice for high specificity amplification and provides robust amplification of a wide variety of amplicons, regardless of GC content." EX1121.002; *see also id.* at .001 ("With the highest fidelity amplification available (>100 times higher than Taq), Q5 DNA Polymerase results in ultra-low error rates.").

D. Sequencing

78. DNA sequencing is a process wherein the sequence of bases in DNA (*i.e.*, the order of bases in a DNA strand) is determined.

79. In 1977, Frederick Sanger developed a dideoxy chain termination method of DNA sequencing (now called Sanger sequencing). EX1070.001-.003. Sanger sequencing is based on parallel polymerase extension reactions, with each incorporating a different dideoxynucleotide analog (*i.e.*, of the A, C, G, and T bases) into extending primer strands, where incorporation of a dideoxynucleotide analog prevents addition of a further nucleotide to a DNA strand, terminating extension.

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Id. The resulting chains can be separated based on size using gel electrophoresis to determine the sequence. *Id.* The amount of nucleotide sequences that could be determined was limited, and the cost was relatively high. *See id.* at .003-.005.

80. In the early 2000s, Next Generation Sequencing ("NGS"), also called High Throughput Sequencing ("HTS"), technologies emerged that allowed for higher throughput of DNA and lower cost. EX1077.002; EX1124 at Abstract. By approximately 2008, HTS was commercially available, including through the Roche/454 FLX, Illumina/Solexa Genome Analyzer and Applied Biosystems SOLiD System. EX1077.002; EX1103.002. By at least 2013, "[a]dvances in sequencing technologies ha[d] enabled the rapid identification of somatic genomic alterations in individual tumors." EX1032.002.

81. NGS uses massively parallel sequencing of many fragments of DNA. Parallel sequencing allows the processing of millions of DNA molecules in parallel, producing millions of "sequencing reads." EX1103.002. These sequence reads, the individual DNA base sequences, must then be aligned (mapped) so that the sequences cover the full region of interest, thus providing the sequence of the full region rather than disparate parts of the region. This is achieved through alignment programs, which match up the overlapping parts of the individual reads into a cohesive DNA sequence for that region. *Id.* at .008. Sequencing platforms that

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carry out NGS use a variety of sequencing and detection biochemistry but generally have a common workflow to produce these sequence reads. *Id.* at .002.

82. As mentioned, DNA amplification techniques, such as PCR, may be used in conjunction with DNA sequencing techniques and can tremendously increase the accuracy of sequencing. Essentially, increasing the copies of DNA for sequencing provides an increased number of sequencing reads, with higher "sequencing depth" or "read depth," increasing accuracy by increasing the statistical significance of the obtained consensus sequence. EX1056.005 ("SNP discovery increases with increasing depth"). Multiplex PCR allows for the efficient amplification of multiple loci in a sample (*see supra* section VI.C.), which in turn allows for greater read depth of any one locus of interest. This is why multiplex PCR is important for sequencing, especially with respect to small amounts of DNA, such as cfDNA samples, increasing the accuracy of sequencing:

> To give a confidence level of how accurate the sequencing information is, the term 'depth' is used to define the average number of times each nucleotide in the genome is observed. For example, if each nucleotide of the genome is reported an average of 10 times during sequencing, a depth level of 10× is obtained. Read depth is important to interpret structural variations, because for a given interval, an increase in the amount of reads at a given read depth may indicate an increase in copy number, whereas a decrease in the amount of reads at a given read depth may indicate deletions (Figure 3). In general, as the read depth level increases, the sequencing information becomes more confident.

See EX1061.004.

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83. HTS technologies were in regular use to perform whole genome sequencing ("WGS"), which is a comprehensive sequencing method used to analyze entire genomes. EX1061.004 ("The order of DNA sequence and its variation dictates human developmental processes, uniquely identify each person, and encodes our susceptibility to diseases. By using high throughput DNA sequencing (DNA-seq) technologies, it is possible to identify genetic variants that play a role in human health. In whole genome sequencing, sequencing information of both the exons and introns is obtained, which may provide critical information on enhancer regions, promoters, and cis/trans regulatory elements that reside in the intronic regions along with structural variants such as copy number variants (CNV), inversions, and translocations affecting the exonic regions."). A related technique, called whole exome sequencing ("WES"), sequences just the protein-coding regions in a genome.

84. WGS and WES were relatively simple with HTS by 2015. The '454 patent describes that Illumina and Ion Torrent products that performed whole exome sequencing were available. EX1001 at 167:66-168:19 ("Each biopsied sample was assayed by whole exome sequencing (Illumina HiSeq200; Illumina, San Diego, Calif.), followed by AmpliSeq® sequencing (Ion Torrent, South San Francisco, Calif.)."). Commercial products were widely available for use in WGS and WES. EX1032.002 ("tagged-amplicon deep sequencing for *PIK3CA* (encoding the

phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha protein) and *TP53* (encoding tumor protein p53) or paired-end whole-genome sequencing (see the Supplementary Appendix). Tagged-amplicon deep sequencing was done by means of the Fluidigm Access Array and sequencing on the Illumina GAIIx or HiSeq instruments. Paired-end sequencing was done with the use of the Illumina HiSeq2000 instrument.").

85. Sequencing was also well-known in the art to be useful for identifying SNVs in cancer patients. EX1059.013.

86. By April 2015, many factors, including the introduction and refinement of the NGS methods mentioned above, had led to an exponential decrease in DNA sequencing costs, with the cost to sequence one human genome falling from around sixteen million dollars in 2005 to around five thousand dollars in 2014. EX1119; EX1123; EX1086.006 ("[S]equencer speeds have improved dramatically in just the past several months, and this trend is likely to continue. Thus, it does not seem that cost and time will remain significant impediments for much longer.").

E. Minimal Residual Disease (MRD) Monitoring

87. Minimal residual disease ("MRD") monitoring, as applicable in this declaration, is the monitoring of residual cancer after a patient has gone through some nature of treatment, such as surgery, radiation, or chemotherapy. EX1130.002 ("The analysis of circulating cell-free tumor DNA has remarkable

potential for the detection and monitoring of cancers. Genetic alterations are present in virtually all types of cancers, allowing broad applicability of this approach regardless of the primary organ and histologic type of the cancer."). MRD monitoring was developed to provide patient-specific care by evaluating a specific individual's tumor, that tumor's susceptibility to different treatments, and the individual's remission or likelihood of remission. EX1059.012-.013; EX1003.001. Rather than relying on wholesale chemotherapy and radiation treatments without consideration of tumor sequence, MRD monitoring allows identifying and designing treatments more likely to be effective for a specific patient's cancer. EX1037.001-.002; EX1003.001 ("Characterization of tumor mutation profiles is required for informed choice of therapy, given that biological agents target specific pathways and effectiveness may be modulated by specific mutations"); *see* EX1004.001-.002 ("The concept of *intra*tumoural heterogeneity has long been blamed for treatment failure in ovarian carcinoma and other primary cancers....As such, the divergence of clonal cell populations confers a heterogeneous genomic tapestry with implications for clinical endpoints, including the acquisition of metastatic potential [17–21] and chemotherapeutic resistance [22,23]."). As detailed herein, MRD monitoring was routinely conducted as of 2013.

88. MRD monitoring generally follows two subsets of steps: 1) WGS or WES of the cancer/tumor and, optionally, the patient's healthy cells to obtain

sequence data and 2) follow-up cfDNA sequencing. EX1003.002-.003; EX1004.001-.002. Essentially, this process finds differences between the healthy DNA and the cancerous DNA and compares the cfDNA with the cancerous DNA to look for remission or "tumor burden," which can show treatment effectiveness and the size or advancement of the tumor. EX1003.002-.003, .010. Through this process, different treatments can be used until an effective combination is found, without the need to look for a recurrent tumor, which can involve very invasive surgeries. *Id.*

89. The first step in MRD monitoring is determining the mutations, commonly SNVs, specific to tumor DNA. EX1003.002; EX1004.001. To that end, a plethora of studies have conducted WES and WGS of cancers, usually from a tumor biopsy, in patients. EX1003; EX1004; EX1032.002 (teaching the use of whole genome sequencing for identification of somatic genomic alterations).

90. The next step in MRD monitoring, after treating the cancer, is to isolate cfDNA from plasma and determine whether tumor DNA is present. The presence of tumor DNA can be determined by using PCR amplification on the specific mutated sequences in the cancer DNA and subsequently sequencing the amplified DNA to look for such mutations. EX1003 at Fig. S1, S3; EX1032.002 ("Studies have shown the feasibility of using circulating tumor DNA to monitor tumor dynamics in a limited number of patients with various solid cancers, but few cases

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of breast cancer have been analyzed. Here, we provide a direct comparison between circulating tumor DNA and other circulating bio-markers (CA 15-3 and circulating tumor cells) and medical imaging, the current standard of care, for the noninvasive monitoring of meta-static breast cancer.").

91. An example of this is shown below:

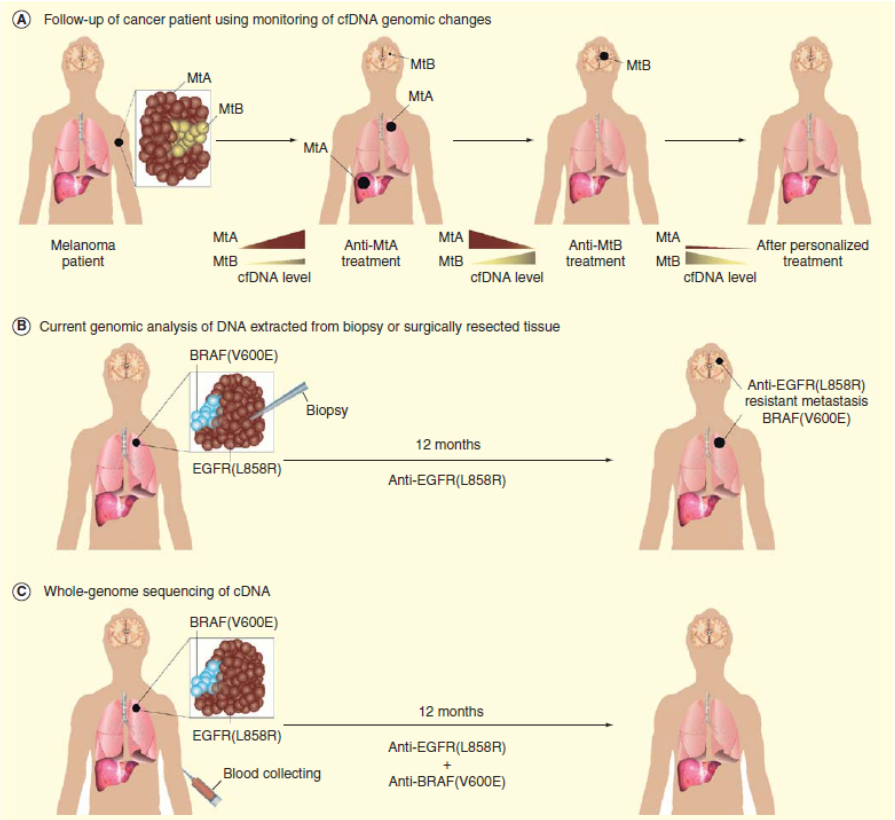

Figure 2. Potential advantages and applications of cfDNA analysis. (A) Application of cfDNA for patient treatment follow-up. In this example, a melanoma patient presents a heterogeneous primary tumor constituted by mutation profile A (MtA) and mutation profile B (MtB). The cfDNA assessment indicates a higher level of MtA as a consequence of being the predominant tumor subclone. After starting with the treatment, anti-MtA clones level in cfDNA decreased while MtB is starting to be detected and specific therapy is selected. **(B)** Current treatment decision based on genetic or genomic analysis of biopsies or sections obtained from surgically resected tumor tissues. In this example, the resected tumor of a patient with non-small cell lung carcinoma is analyzed revealing targetable mutations in *EGFR* gene. After one year of treatment, the patient relapses with a tumor harboring *BRAF* (V600E) mutation. **(C)** The application of WGS of cfDNA can identify the genetic mutation of the predominant clones (*EGFR* (L858R)) and the under-represented subclones harboring *BRAF* (V600E). In this diagnostic setting, both targetable mutations could be considered for treatment selection.

EX1037.007 (Figure 2).

92. As treatment continues, plasma samples can be taken over time, and the cfDNA contained within may be analyzed to continue monitoring tumor burden or recurrence. EX1003 at Abstract; EX1059.013; EX1032.001-.011; EX1075.001-.002.

93. Many different techniques have been used to monitor cfDNA, including: shotgun sequencing –

> One can also envision the possibility of using shotgun sequencing of a plasma sample upon presentation of a cancer patient, mining the shotgun sequencing data of plasma DNA for copy number aberrations and SNVs, and then specifically targeting such sequences for serial monitoring purposes. In this regard, solution-phase hybridization-based capture approaches have already been successfully used to enrich plasma DNA for noninvasive prenatal diagnosis (34, 35). Hence, we see that the targeted and shotgun approaches can be used in combination for cancer detection and monitoring.

(EX1059.013);

TAm-Seq –

> Plasma of cancer patients contains cell-free tumor DNA that carries information on tumor mutations and tumor burden. Individual mutations have been probed using allele-specific assays, but sequencing of entire genes to detect cancer mutations in circulating DNA has not been demonstrated. We developed a method for tagged-amplicon deep sequencing (TAm-Seq) and screened 5995 genomic bases for low-frequency mutations. Using this method, we identified cancer mutations present in circulating DNA at allele frequencies as low as 2%, with sensitivity and specificity of >97%. We identified mutations throughout the tumor suppressor gene TP53 in circulating DNA from 46 plasma samples of advanced ovarian cancer patients. We demonstrated use of TAm-Seq to noninvasively identify the origin of metastatic relapse in a patient with multiple primary tumors. In

> another case, we identified in plasma an EGFR mutation not found in an initial ovarian biopsy. We further used TAm-Seq to monitor tumor dynamics, and tracked 10 concomitant mutations in plasma of a metastatic breast cancer patient over 16 months. This low-cost, high-throughput method could facilitate analysis of circulating DNA as a noninvasive "liquid biopsy" for personalized cancer genomics.

(EX1003 at Abstract); and

> chromosomal rearrangements –
>
> Tumor-specific (somatic) chromosomal rearrangements have the potential to serve as highly sensitive biomarkers for tumor detection. Such alterations are not present in normal cells and should be exquisitely specific. Rearrangement-associated biomarkers therefore offer a reliable measure that would be useful for monitoring tumor response to specific therapies, detecting residual disease after surgery, and long-term clinical management. Recurrent somatic structural alterations, such as those involving the BCR-ABL oncogene (the target of the Philadelphia chromosome translocation), immunoglobulin genes, T cell receptor genes, and the retinoic acid receptor a gene, have been shown to be useful as diagnostic markers in certain hematopoietic malignancies (16–20). However, recurrent structural alterations do not generally occur in most solid tumors. We reasoned that any structural alteration identified in an individual's tumor could be used for analogous purposes, whether it was found in tumors of the same type in other individuals and whether it was a "driver"—causing a selective growth advantage—or a "passenger." We describe herein our efforts to implement this concept in representative examples of common solid tumors.

(EX1075.002).

F. Clonal Heterogeneity

94. One challenge that has been documented in MRD monitoring is the heterogeneity of tumors and tumor DNA. As discussed above, DNA mutations

cause cancer. *See supra* Section VI.B. In a healthy cell, there are numerous checks that control cell growth and division. "Cancers arise from a single ancestral cell that has acquired enough somatic mutations to deregulate its own proliferation and expand into a large cell population with clonal mutations matching the ancestral profile." EX1039.001; *see also id.* ("Loss of function in genes for genome stability and repair establishes a mutator phenotype in which a greater rate of somatic mutation provides the cell population with a vast repository of mutations in low copy."); *see also* EX1004.002 ("Rooted in the clonal evolution model of tumor progression, cancers are assumed to originate from a monoclonal composition (the ancestral clone), with subsequent evolution leading to selective expansions of genomically distinct subclones [12] with distinct phenotypic properties [12-16]."). Cells with mutation(s) may undergo apoptosis, or a process of pre-programmed cell death. Cancerous cells, however, have mutations that allow the cells to both avoid the checks on growth and division and to avoid apoptosis. EX1126.010; *see also id.* at .002 ("Irradiation or drugs used for cancer chemotherapy results in DNA damage in some cells, which can lead to apoptotic death through a *p53*-dependent pathway."); *see also id.* at .006 ("Once in the cell, granzyme A activates DNA nicking via DNAse NM23-H1, a tumor suppressor gene product (Fan et al., 2003). This DNAse has an important role in immune surveillance to prevent cancer through the induction of tumor cell apoptosis."); EX1039.001; EX1064 at Abstract ("In

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particular, mutations in the gene *PIK3CA* that encodes the p110α catalytic subunit of the PI3K enzymes result in cell proliferation and resistance to apoptosis *in vitro* and induce breast tumors in transgenic mice.").

95. Each time a cancerous cell divides and grows, there is a chance it will accumulate more DNA mutations without undergoing apoptosis. EX1126.010; EX1039.001. Accordingly, a cell may accumulate mutations not shared with other cells in the tumor, i.e., "subclonal" mutations. *Id.* A mutation present in the ancestor cancerous cell, an ancestral mutation, is a "clonal" mutation, while a mutation present in only a subpopulation of the cancer cells is a "subclonal" mutation. *Id*. This variety in the tumor cells' mutations is "clonal heterogeneity." *Id*.

96. Heterogeneity of mutation (*i.e.*, tumor heterogeneity) has been linked to treatment resistance and recurrence of cancer. *See, e.g.*, *id.*; EX1005.006 ("mutational heterogeneity may be able to explain resistance mechanism of EGFR-TKI"); EX1004.002. While one sub-clone of the cancer may be susceptible to a treatment, another sub-clone may be resistant to that treatment. *Id.* Thus, as treatment continues, treatable sub-clone(s) may die off, while resistant sub-clone(s) do not and continue to propagate. *See* EX1039.001 ("Those mutations conferring a selective advantage preferentially expand in number, forming subpopulations with the same subclonal mutations.").

97. Tracking and measuring heterogeneity can be done using the same techniques as for sequencing the genome of any cancer, as discussed above. *See supra* Sections VI.D, VI.E; *see also* EX1004.002. cfDNA can also be used to track heterogeneity. EX1004.002 ("examination of plasma ctDNA suggested that representative mutations in the ancestral clone and mutations present in subclones could be detected in ctDNA.").

VII. PRIOR ART REFERENCES

A. Forshew

98. Forshew (including its supplementary material) was publicly available online as of May 30, 2012. EX1003. In my experience, articles published in scientific journals, including any supplemental materials, are publicly available online as of the date that they publish. The right-hand margin of the body of the article also states "[d]ownloaded from stm.sciencemag.org on June 6, 2012." EX1003. EX1003 appears to be an authentic copy of Forshew downloaded from the publisher's website (www.ScienceTranslationalMedicine.org). Forshew was also cited as prior art during prosecution of the '454 patent, and the Applicants did not contest its status as prior art. EX1022.

99. Forshew discloses methods to identify tumor-specific SNVs in a patient's tumor sample and monitoring those SNVs in the patient's blood plasma samples for relapse detection. EX1003.002-.010. Forshew explains that

"[e]volutionary changes within the cancer can alter the mutational spectrum of the disease and its responsiveness to therapies," which motivated the development of "[s]ensitive methods for detecting cancer mutations in plasma [that] may find use in early detection screening, prognosis, monitoring tumor dynamics over time, or detection of minimal residual disease." *Id.* at .002.

100. Forshew teaches tagged-amplicon deep sequencing ("TAm-Seq"), which is a specific method for detecting low frequency mutations, as well as the use of TAm-Seq to identify and monitor cancer mutations in patients. EX1003.002, Abstract. Below is a general description of the steps of the TAm-Seq method disclosed in Forshew.

101. First, tumor-specific mutations or SNVs in cfDNA associated with cancer or tumors in a patient were identified. EX1003.003, .010. This was accomplished by performing whole genome sequencing of both healthy and tumor tissue and subsequently comparing the sequencing data to identify tumor-specific mutations or SNVs. *Id.* at .010 ("[w]hole-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"), Table S7 legend ("genomic libraries from tumour and matched normal tissues were prepared").

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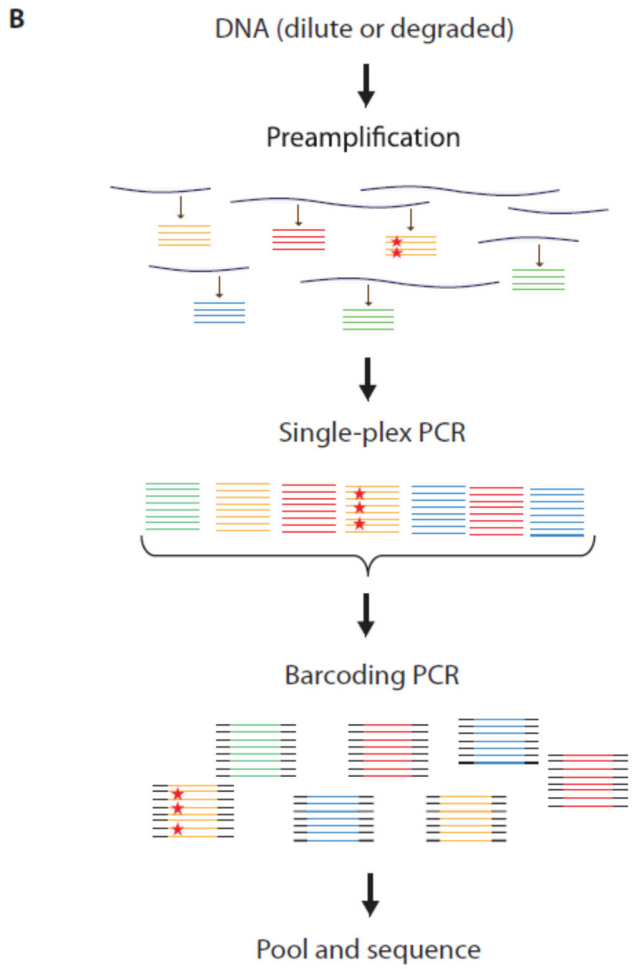
102. Next, primer pairs were designed for the amplification of ten different genetic loci encompassing the tumor-specific mutations or SNVs identified in the previous step. EX1003.010, Table S2.

103. Then, a targeted multiplex PCR reaction (*i.e.*, "Pre Amp") was performed on a cfDNA sample from the cancer patient using target-specific primers for the amplification of the different genetic loci. Specifically, in the targeted multiplex PCR reaction "all primer sets were used together to capture the starting molecules present in the template." EX1003.004. The targeted multiplex PCR reaction was followed by a single-plex PCR reaction that further purified the DNA. *Id.* at .004, .010. This was then followed by an additional amplification step that introduced barcodes and sequencing-adapters. *Id.* at .003-.004, .010.

104. Finally, the amplified cfDNA was sequenced using NGS and a bioinformatics analysis was performed on the resulting sequencing data to determine whether the cfDNA included the tumor-specific mutations or SNVs. EX1003 at Table S4, Fig. S1, legend.

105. A depiction of the basic overall workflow of the TAm-Seq method is shown in Figure 1(B), reproduced below:



EX1003.003, Fig. 1(B).

106. Figure 1(B) is described as follows:

Multiple regions were amplified in parallel. An initial preamplification step was performed for 15 cycles using a pool of the target-specific primer pairs to preserve representation of all alleles in the template material. The schematic diagram shows DNA molecules that carry mutations (red stars) being amplified alongside wild-type molecules. Regions of interest in the preamplified material were then selectively amplified in individual (single-plex) PCR, thus excluding nonspecific products. Finally, sequencing adaptors and sample-specific barcodes were attached to the harvested amplicons in a further PCR.

EX1003.003 (Fig. 1(B) legend).

B. Bashashati

107. Bashashati was publicly available online as of 2013 (including its supplemental materials). EX1004. In my experience, articles published in scientific journals are publicly available online as of the date that they publish. Bashashati states it was "[p]ublished online in Wiley Online Library," has a citation indicating publication in 2013 ("J Pathol ***2013***; 231: 21-34" (emphasis added)), and states "Received | May 2013; Revised 4 June 2013; Accepted 7 June 2013." *Id.* The right-hand margin of each page also states "10969896, 2013, 1, Downloaded from https://pathsocjournals.onlinelibrary.wiley.com/doi/10.1002/path.4230 by Test, Wiley Online Library on [15/09/2023]." *Id.* All of this information is wholly consistent with publication in 2013. *Id.* It also appears to be an authentic copy of Bashashati downloaded from wileyonlinelibrary.com. *Id.* at footer. Bashashati was also of record during the examination of the '454 patent, as it is listed on the face of the '454 patent, even if not applied in a rejection. EX1001.009; EX1005; EX1022.

108. Bashashati teaches sequencing exomes of tumor tissue and healthy tissue, and comparing the sequence data to identify tumor-specific mutations, which are then detected in the cfDNA of plasma samples. EX1004.005-.009.

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109. Bashashati discloses performing sequencing on various regions of an individual tumor. *Id.* at .002. Bashashati discloses that the "concept of intratumoural heterogeneity ha[d] long been blamed for treatment failure in ovarian carcinoma and other primary cancers." *Id.* Thus, Bashashati was motivated to perform "exome sequencing,…targeted amplicon deep sequencing and gene expression profiling on 31 spatially and temporally separated…tumour specimens," which resulted in "highly individual evolutionary trajectories and diverse genomic tapestries prior to therapy, exposing an essential biological characteristic to inform future design of personalized therapeutic solutions and investigation of drug-resistance mechanisms." *Id.* at Abstract.

110. Specifically, Bashashati discloses that "[t]issue was obtained from a total of 31 tumour sites…matched normal DNA for each patient (Table 1)." EX1004.002.

111. Bashashati further discloses identifying tumor-specific mutations, including SNVs, by using targeted amplification and sequencing, stating that "tumour samples were used as index discovery samples to identify sets of mutations for interrogation by deep amplicon sequencing…to: (a) confirm predicted mutations; (b) determine the presence/absence of mutations in specific samples; and (c) infer clonal diversity estimates both within and between samples from the same patient." *Id.*

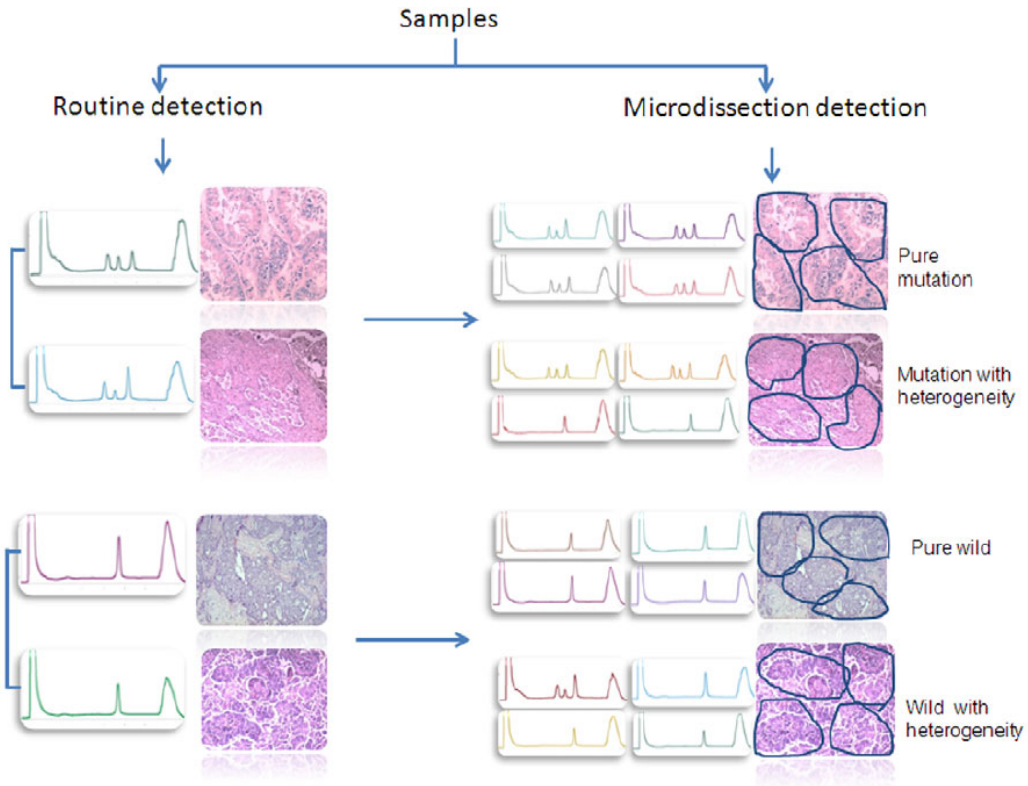
112. Bashashati then discloses targeted amplification and sequencing of target loci with SNVs from ctDNA from plasma "to identify the presence of clonally dominant and subclonal mutations through deep sequencing of ctDNA." *Id.* For four patients, selected clonal and subclonal variants were identified in these exomes and subsequently assayed in plasma. *See, e.g.*, *id.*; *id.* at .007-.008 ("The FT lesion in case 5 harboured seven mutations that were not present in all samples, indicating that the clones comprising case 5h were unlikely to have been ancestral to the tumours. Rather, phylogenetic analysis suggested that case 5h had closer clonal relationships with case 5b and 5f (left ovary and para-aortic lymph node, respectively) compared to cases 5a, 5d, 5e and 5g (Figure 1D).…Results from *PIK3CA* mutation sequencing suggested the FT lesion in case 4 was composed of heterogeneous subclones.").

C. Bai

113. Bai was publicly available online as of February 13, 2013. EX1005. In my experience, articles published in scientific journals, including any supplemental materials, are publicly available online as of the date that they publish. Bai states it was "**Published** February 13, 2013" and recites "February 2013 | Volume 8 | Issue 2 | e54170," which is wholly consistent with publication on that date. *Id.* (emphasis added). It also appears to be an authentic copy of Bai downloaded from www.plosone.org. *Id.* at footer. Bai was also of record during

the examination of the '454 patent, as it is listed on the face of the patent, even if not applied in a rejection. EX1001.009; EX1005; EX1022.

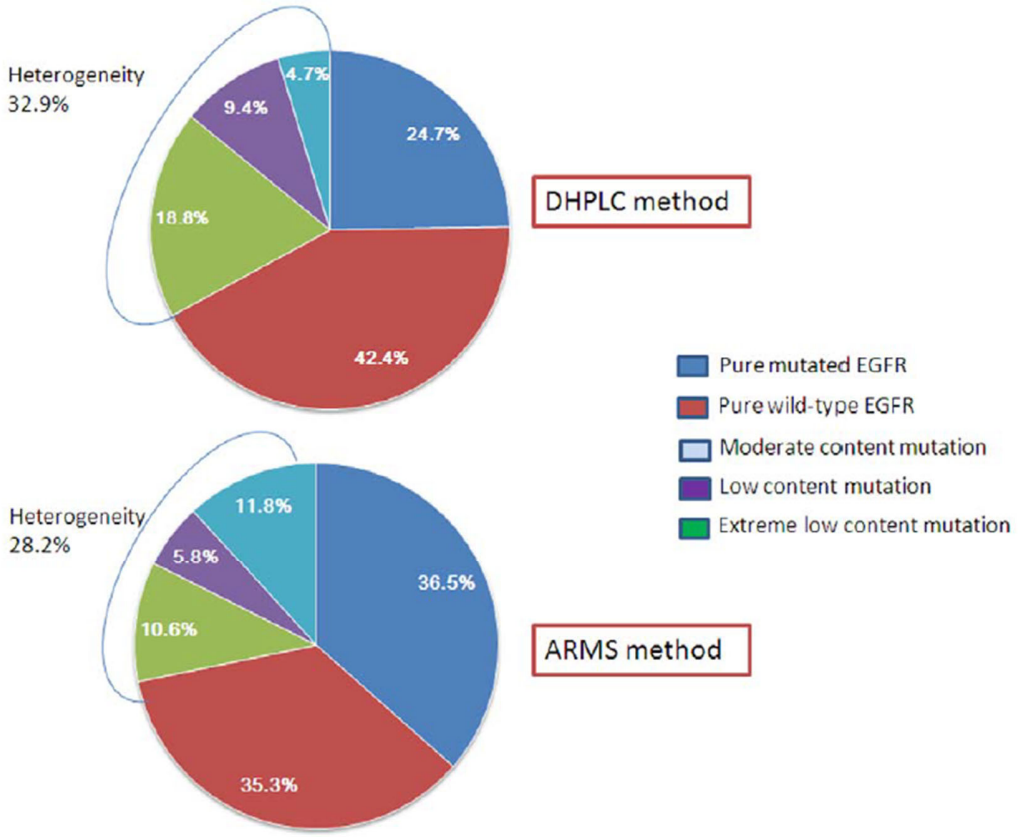
114. Bai teaches two methods for determining heterogeneity in different portions of the same tumor sample, in multiple samples, specifically for mutations in the *EGFR* gene, a known tumor suppressor gene. EX1005.002-.003, Fig. 1, Fig. 2. Heterogeneity in the *EGFR* gene within a tumor is shown in Figure 2:



Id. at Fig. 2. ("A representative example of intratumoral *EGFR* genetic heterogeneity").

115. Specifically, Bai discloses using "Denaturing High Performance Liquid Chromatography (DHPLC) and Amplification Refractory Mutation System

(ARMS)" in order to determine mutation frequencies within a tumor sample. *Id.* at Abstract, .001-.002. Bai determined that "[m]utant frequencies varied widely throughout individual tumors, ranging between 5%-100% by DHPLC and 1%-100% by ARMS." *Id.* at .003. Bai also discloses that a percentage of "samples were identified to carry intratumoral *EGFR* mutational heterogeneity." *Id.* These are shown in Figure 1:



Id. at Fig. 1.

116. Further, Bai discloses that "[t]he most important clinical significance of *EGFR* mutational heterogeneity may be able to explain resistance mechanism of

EGFR-TKI." *Id.* at .006. Additionally, this confirmed heterogeneity led Bai to determine that "single point or single time biopsy might not be [the] optimal method to determine personalized EGFR-TKI therapy," instead concluding that a combination of "histological and genetic approaches using information on survival and response profiles" would provide better disease prevention. *Id.*

D. Bentley

117. Bentley is Patent Application Publication No. 2010/0261189 A1, which published October 14, 2010. EX1057.

118. Bentley discloses "a method of investigating by massively parallel sequencing nucleic acids replicated by polymerase chain reaction (PCR), for the identification of mutations and polymorphisms of both known and unknown sequences." *Id.* ¶ 2. Bentley's method further discloses a relationship between the "Number of Reads" and the "Minimum frequency of SNP in population detectable with 95%…[and] 99% confidence." *Id.* ¶ 88. Specifically, sequencing a greater number of reads allows for detecting lower frequency SNVs. *Id.* ¶ 85. Bentley shows the results of this method in Table 2:

NEOGENOMICS EX.1002.058

TABLE 2

SNP Classes	Number of Reads	Minimum frequency of SNP in population detectable with 95% confidence	Minimum frequency of SNP in population detectable with 99% confidence
1	200000	0.002%	0.003%
2	100000	0.005%	0.007%
5	40000	0.014%	0.018%
10	20000	0.028%	0.037%
50	4000	0.14%	0.18%
100	2000	0.28%	0.37%
200	1000	0.55%	0.74%
500	400	1.39%	1.85%
1000	200	2.76%	3.64%

EX1057 ¶ 88 (Table 2). Bentley shows that sequencing to a depth of 40,000 would detect SNPs between a frequency of 0.014% and 0.018%, with 95-99% confidence. *Id*. Bentley also reflects a POSA's understanding that a sequencing depth of 100,000 would detect SNPs between a frequency of 0.005% and 0.007%, with 95-99% confidence. *Id*.

E. Narayan

119. Narayan was publicly available online as of July 15, 2012. EX1086. In my experience, articles published in scientific journals, including any supplemental materials, are publicly available online as of the date that they publish. Narayan recites "Cancer Res; 72(14) July 15, 2012," which is wholly consistent with publication on that date. *Id*. It also appears to be an authentic copy of Narayan

"[d]ownloaded from http://aacrjournals.org/cancerres/article-pdf/72/14/3492/2671690/3492.pdf." *Id*. Narayan was also of record during the examination of the '454 patent, as it is listed on the face of the patent, even if it was not applied in a rejection. EX1001.022; EX1086; EX1022.

120. Narayan teaches a "deep sequencing method that demands redundancy within each clonal sequence to produce extremely high quality base calls in short, mutation-prone regions of plasma DNA." EX1086.001. This method was designed to improve next generation sequencing techniques in calling mutant variants found circulating in plasma cfDNA. *Id*. The depth of sequencing disclosed extends to "a median depth of 108,467 reads per locus" and the base call quality to "reduc[ing] the frequency of miscalls…to a median value of 0.014%." EX1086.003-.004.

VIII. CLAIM CONSTRUCTION

A. "Targeted Multiplex Amplification"

121. Claims 1, 8-11, 14, and 22-23 of the '454 patent recite "targeted multiplex amplification." Specifically, independent claims 1 and 14 both recite "performing targeted multiplex amplification...to amplify target loci." EX1001 at claims 1, 14. The '454 patent states that "Amplification refers to a method that increases the number of copies of a molecule of DNA or RNA." EX1001 at 34:52-53. The '454 patent further states that:

> Selective Amplification may refer to a method that increases the number of copies of a particular molecule of DNA (or RNA), or molecules of DNA (or RNA) that correspond to a particular region of DNA (or RNA). It may also refer to a method that increases the number of copies of a particular targeted molecule of DNA (or RNA), or targeted region of DNA (or RNA) more than it increases non-targeted molecules or regions of DNA (or RNA). Selective amplification may be a method of preferential enrichment.

Id. at 34:54-62. The '454 patent further states that "Multiplexed PCR allows simultaneous amplification of many targets in a single reaction." EX1001 at 163:16-17. In view of the foregoing, I construe the term "targeted multiplex amplification" to mean simultaneously "increasing the number of copies of" "many" "particular targeted molecule[s] of DNA (or RNA), or targeted region[s] of DNA (or RNA)" "in a single reaction." Moreover, a POSA would understand that these "copies" that result from amplifying, such as in a multi-step PCR process, are not just the copies that result from the initial copying of the original sample DNA strand, but also the copies of those copies and any subsequent copies. *See* EX1052 at 110:5-111:12 (Dr. Metzker testifying at deposition that copies of original strands and copies of copies are just copies).

B. "Amplicons" and "Sequencing The Amplicons"

122. Claims 1, 8-11, and 13-14 of the '454 patent recite "amplicons" and claims 1 and 14 of the '454 patent recite "sequencing the amplicons." Specifically, independent claims 1 and 14 both recite "performing targeted multiplex

NEOGENOMICS EX.1002.061

amplification…to obtain amplicons" and "sequencing the amplicons." EX1001 at claims 1, 14. Both claims 1 and 14 further recite that the "targeted multiplex amplification" amplifies target loci "to obtain amplicons" and that the "target loci are amplified together in the same reaction volume." *Id.* I understand that Patent Owner interprets this claim element to encompass the sequencing of products that have been subject to additional amplification processes following the "targeted multiplex amplification," such as a PCR process to add barcodes to the amplicons that emerge from the "targeted multiplex amplification."

IX. THE CLAIMS OF THE '454 PATENT WOULD HAVE BEEN OBVIOUS

A. The Prosecution History Does Not Alter My Obviousness Opinions

123. The Patent Office considered Forshew during examination of the '454 patent, and I understand that Patent Owner may contend this shows that the claims are not invalid in view of Forshew.

124. Having considered the record, the fact that the '454 patent issued with Asserted Claim 1 from the Patent Office over rejections grounded on Forshew does not support the validity of claim 1, but rather error in allowance of the claim. As I explain below, the Examiner made two errors.

NEOGENOMICS EX.1002.062

125. First, the Examiner accepted erroneous arguments by Patent Owner regarding whether Forshew discloses multiplex amplification. In response to a rejection in view of Forshew, Patent Owner argued as follows:

> Furthermore, Forshew's TAm-Seq process was rationally and deliberately designed to rely on single-plex, "*individual amplification to purify and select for intended targets*" (pg. 3). In fact, Forshew had considered but dismissed multiplex targeted amplification – "*Multiplex amplification using a large set of primers could result in nonspecific amplification products and biased coverage*" (Fig. 1B, pg. 3). Forshew deliberately resorted to parallel single-plex amplification on Access Array, which physically separate individual primer pairs and individual reaction volumes to avoid issues such as nonspecific amplification and primer dimer formation. There was no reason or motivation for one of ordinary skill in the art to alter Forshew's TAmSeq process by substituting a step rationally and deliberately designed by Forshew for purpose of addressing the issue of nonspecific amplification, for a step already considered but dismissed by Forshew.

EX1022.004 (emphasis in original). Thus, Applicant argued that Forshew did not utilize multiplex PCR. In view of these and the other arguments that Patent Owner made, the Examiner allowed the claims. EX1024.003.

126. Yet, Patent Owner's arguments, which the Examiner accepted, that Forshew does not teach multiplex amplification were erroneous. As explained below, the preamplification process in Forshew is in fact a multiplex amplification and Patent Owner's own expert has admitted this in a pending district court proceeding. *See infra* Section IX.B.1.c.

NEOGENOMICS EX.1002.063

127. Second, the Examiner appears to have misapprehended or overlooked that increasing sequencing depth would have been obvious to a POSA before 2015.

128. During prosecution of the '454 patent, the Examiner issued a Non-Final Rejection, rejecting some of the then-pending claims as obvious over Forshew and other prior art references. EX1020.005-.009. Then-pending claim 1 stood rejected over the combination of Forshew and Benesova. EX1020.005-.007. Then-pending claim 2, adding the limitation "wherein the high-throughput sequencing has a depth of at least 50,000 per target locus," was not the subject of rejection over the prior art. EX1125.001; EX1020.005-.009. As I explain herein, the use of such sequencing depth is not inventive, and it seems likely the Examiner simply overlooked this claim element.

129. Nevertheless, Patent Owner responded to the obviousness rejection of claim 1 by amending claim 1 to incorporate a depth of sequencing limitation like that in then-pending claim 2. EX1022.003. Additional amendments to claim 1 addressed a Claim Objection and "alleged indefiniteness" (EX1022.002), and Patent Owner argued that these amendments are immaterial to the question of obviousness (EX1022.001-.002).

130. Patent Owner argued for allowance of independent claim 1, over the combination with Forshew, on the basis of the limitation to a sequencing depth of at least 50,000 per target locus:

> Claim 2 stands free of the subject rejections. For the sole purpose of expediting prosecution and without acquiescing to the rejection, Applicant has amended independent claim 1 to incorporate the limitation of previous claim 2, thereby rendering the rejections moot. None of the cited references teaches or suggests sequencing each of the target loci with a depth of read of at least 50,000 per target locus. For at least these reasons, Applicant respectfully request withdrawal of the subject rejections against claims 1 and 3-14.

EX1022.003.

131. The Examiner then withdrew the obviousness rejection of claim 1 over Forshew and Benesova set forth in the Non-Final Office Action mailed "07 July 2022…in view of the arguments and amendment received 07 October 2022." EX1024.006.

132. As discussed above, however, sequencing to "a depth of read of at least 50,000 per target locus" would have been obvious to a POSA before 2015, based on the teaching in Forshew, as well as on sequencing depth more generally being a known factor for increasing sensitivity. *See infra* Section IX.B.1.d. Indeed, as I explain below, Forshew expressly teaches increasing sequencing depth so as to increase sensitivity. *See infra* Section IX.B.1.d.i.

133. It is my opinion, accordingly, that the Examiner misapprehended or overlooked that Forshew would have taught or suggested sequencing with "a depth of read of at least 50,000 per target locus."

NEOGENOMICS EX.1002.065

B. Claims 1-6, 8-20, and 22-28 of the '454 Patent Are Obvious Over Forshew In View Of A POSA's Knowledge

134. As described below, it is my opinion that claims 1-6, 8-20, and 22-28 of the '454 patent are unpatentable as obvious over Forshew in view of a POSA's knowledge.

1. Claim 1:

a. [1.preamble]: A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample, the method comprising:

135. To the extent that the preamble of claim 1 is limiting, Forshew discloses a method of preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more SNV mutations in the plasma sample.

136. Forshew discloses preparing a plasma sample of a subject having cancer and performing TAm-Seq to detect SNV mutations in the plasma sample. For example, Forshew discloses that the "[p]lasma of cancer patients contains cell-free tumor DNA that carries information on tumor mutations and tumor burden." EX1003 at Abstract. Forshew further discloses the "use of TAm-Seq to noninvasively identify the origin of metastatic relapse in a patient with multiple primary tumors." *Id.*

NEOGENOMICS EX.1002.066

137. Specifically, Forshew discloses performing whole genome sequencing to identify tumor mutations, including SNVs, in tumor samples, followed by performing TAm-Seq to identify those SNVs in plasma samples:

> 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. 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).

EX1003.010 (emphasis added); *see also id.* at .004 ("We applied TAm-Seq to directly identify mutations in plasma of cancer patients."); *see also* EX1003.015 ("Dilution of plasma DNA samples to determine allele quantification accuracy"), .017-0.19 (Supplementary Methods).

138. Forshew discloses the identification of tumor mutations, specifically SNVs, which were single nucleotide changes or substitutions in a genome. *See* EX1003.010; EX1003.031-.033 (Tables S6-S7).

139. Therefore, Forshew discloses the preamble of claim 1.

b. [1.a]: performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;

140. Forshew discloses to a POSA performing whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations.

141. Forshew discloses performing "[w]hole-genome sequencing of tumor material [] to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy." EX1003.010. This whole genome sequencing was performed using "an Illumina HiSeq 2000 sequencer to 30x genomic coverage" and "the standard Illumina paired-end sample preparation kit according to the manufacturer's instructions." EX1003.033. Table S7 includes the affected gene, genomic location of the amplicon, genomic location of mutation, reference allele, and mutant allele, as depicted below:

NEOGENOMICS EX.1002.068

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

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

EX1003.033.

142. The variants identified are single nucleotide changes (*i.e.*, SNVs), as shown above in the columns titled "Reference allele" and "Mutant allele" in Table S7. A POSA would have known that the genes in table S7 are associated with cancer, and in particular, breast cancer. *See, e.g.*, Sarah-Jane Dawson et al., "A New Genome-Driven Integrated Classification Of Breast Cancer And Its

Implications," 32 EMBO J. 617, 625 (2013) (BUB1 is a "checkpoint, DNA damage repair and apoptosis gene[]" associated with breast cancer.) (EX1131.009); Amaury G. Dumont et al., "The Favorable Impact Of PIK3CA Mutations On Survival: An Analysis Of 2587 Patients With Breast Cancer," 31 Chin. J. Cancer 327, 327 (2012) (*PIK3CA* "regulates a number of cellular processes, including cell survival, cell growth, and cell cycle progression" and is associated with breast cancer) (EX1064); Xiaocong Fang et al., "Genetic Network And Gene Set Enrichment Analysis To Identify Biomarkers Related To Cigarette Smoking And Lung Cancer," 39 Cancer Treatment Reviews 77, 83 (2013) (ARAP3 is a "common involved genes in smoking and lung cancer related networks") (EX1065); P. Andrew Futreal, et al., "A Census of Human Cancer Genes," 4 Nature Reviews 177, Supplementary information S1 (2004) (the MET gene is associated with cancer) (EX1066); Ewelina Rojewska et al., "Expression Profiling of Genes Modulated by Minocycline in a Rat Model of Neuropathic Pain," 10 Molecular Pain at 10 (2014) ("Gimap5…has been shown to be integral to T cell survival and development" and "regulate[s] proapoptotic and antiapoptotic T cell pathways.") (EX1099); Patrícia Henriques Domingues et al., "Association between Inflammatory Infiltrates and Isolated Monosomy 22/del(22q) in Meningiomas," 8 PLOS ONE at 8 (2013) (*ZFYVE21* genes, are "mainly involved in cellular functions related to cell death, cell cycle, cell growth and proliferation, and to cellular assembly.") (EX1063); Shuangge Ma & Jian Huang, "Regularized

Gene Selection In Cancer Microarray Meta-Analysis," 10 BMC Bioinformatics at 7 (2009) ("KIAA0406…constitutes the predictor of PI3 kinase activation. The PI3 kinase signaling pathway is emerging as a promising therapeutic target in a number of cancers") (EX1076); Carmela Ricciardelli et al., "The ADAMTS1 Protease Gene Is Required for Mammary Tumor Growth and Metastasis," 179 Am. J. Pathology 3075 (2011) ("ADAMTS1 may promote mammary tumor growth and progression to metastasis in the PyMT model and is a potential therapeutic target to prevent metastatic breast cancer.") (EX1097).

143. Forshew also discloses identifying tumor mutations, specifically SNVs, by sequencing exons on a tumor sample. EX1003.004 (Validation and sensitivity for mutation identification in ovarian tumor samples); EX1003.015 (Sanger Sequencing). Forshew discloses 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 (36) (Supplementary Methods)." EX1003.004. *TP53* is a tumor suppressor gene associated with cancer, so a POSA would have understood that Sanger sequencing to identify mutations in *TP53* constitutes identification of tumor-specific SNV mutations. EX1003.002. 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)." EX1003.004. These "single-base substitutions" are SNVs. The SNVs were identified by well-known methods of sequencing. For example, Table S3 includes the affected gene, mutation and base change, and whether it was identified by Sanger sequencing, as shown below:

NEOGENOMICS EX.1002.072

Table S3: Mutations identified in FFPE samples. Diagnosis: type (FT, fallopian tube; O, ovarian; PP, primary peritoneal), histiotype (END, endometrioid, grade III; HGSOC, high-grade serous ovarian carcinoma; S/CC, mixed serous and clear cell; S/END, mixed serous papillary and endometrioid, high grade), and stage. FFPE source: sample type (B, biopsy; IDS, interval-debulking surgery; PS, primary surgery), sample tissue (FT, fallopian tube; O, ovary; OM, omentum; P, peritoneum; SC, sigmoid colon; U, uterus). Cellularity: estimated from H&E-stained slide of the FFPE block. Identified by Sanger sequencing: I, identified; FAIL, samples where one duplicate had a technical fault; Indel, indel identified (indels were not analyzed by the calling algorithm); M, missed; U, unsequenced region or sample. S-135 and S-165 were from the same patient, but collected at different timepoints. S-140 and S-106 were from the same block, cut in different batches. S-111, S-143, and S-122 had two mutations called each. S-133, S-169, and S-147 had known indels, and did not have any point mutations called. S-119 had no mutations called. S-176, S-177, and S-178 failed harvesting in one of the repeats and calling algorithm was not applied.

Patient ID	Age at diagnosis	Diagnosis: type, histiotype, and stage.			Sample ID	FFPE source		Cellularity	Gene	Mutation and base change (genome build hg19)		cDNA change	Protein change	TAm-Seq Allele frequency (repeat 1)	TAm-Seq Allele frequency (repeat 2)	Identified by Sanger sequencing
1	60	O	HGSOC	III	S-131	PS	O	60%	*TP53*	17:7577570	C>T	c.711G>A	p.M237I	0.72	0.70	I
2	63	O	HGSOC	III	S-142	IDS	O	40%	*TP53*	17:7578203	C>A	c.646G>T	p.V216L	0.42	0.43	I
3	54	O	HGSOC	III	S-150	IDS	O	70%	*TP53*	17:7577538	C>T	c.743G>A	p.R248Q	0.87	0.91	I
4	46	O	HGSOC	I	S-167	PS	O	70%	*TP53*	17:7578290	C>T	c.560-1G>A	p.?	0.73	0.73	I
5	38	O	HGSOC	III	S-174	PS	O	90%	*TP53*	17:7578268	A>G	c.581T>C	p.L194P	0.69	0.72	I
6	63	O	HGSOC	III	S-102	IDS	FT	60%	*TP53*	17:7578271	T>C	c.578A>G	p.H193R	0.39	0.34	I
7	68	O	HGSOC	III	S-112	IDS	O	60%	*TP53*	17:7578263	G>A	c.586C>T	p.R196*	0.30	0.28	I
8	60	O	HGSOC	III	S-132	PS	O	90%	*TP53*	17:7577120	C>T	c.818G>A	p.R273H	0.74	0.75	I
9	62	PP	HGSOC	IV	S-151	PS	SC	70%	*TP53*	17:7578479	G>A	c.451C>T	p.P151S	0.39	0.38	I
10	58	PP	HGSOC	IV	S-168	IDS	O	90%	*TP53*	17:7578508	C>T	c.422G>A	p.C141Y	0.82	0.86	I
11	55	O	HGSOC	III	S-104	IDS	O	15%	*TP53*	17:7578370	C>T	c.559+1G>A	p.?	0.12	0.12	M
12	62	O	HGSOC	IV	S-115	IDS	O	70%	*TP53*	17:7577579	G>T	c.702C>A	p.Y234*	0.57	0.59	I
13	45	O	HGSOC	IV	S-123	IDS	O	80%	*TP53*	17:7577574	T>C	c.707A>G	p.Y236C	0.53	0.55	I
14	58	O	HGSOC	III	S-153	IDS	O	95%	*TP53*	17:7578212	G>A	c.637C>T	p.R213*	0.56	0.56	I
15	61	O	HGSOC	IV	S-116	IDS	O	98%	*TP53*	17:7577121	G>A	c.817C>T	p.R273C	0.76	0.75	I
16	65	O	HGSOC	III	S-127	IDS	O	90%	*TP53*	17:7577094	G>A	c.844C>T	p.R282W	0.76	0.74	I
17	64	O	HGSOC	III	S-145	PS	FT	60%	*TP53*	17:7578176	C>T	c.672+1G>A	p.?	0.52	0.55	I
18	63	FT	HGSOC	III	S-161	PS	O	95%	*TP53*	17:7578534	C>G	c.396G>C	p.K132N	0.86	0.88	I
19	68	PP	HGSOC	III	S-170	IDS	U	50%	*TP53*	17:7579312	C>A	c.375G>T	p.T125T	0.22	0.20	I
20	67	PP	HGSOC	III	S-108	IDS	OM	20%	*TP53*	17:7578431	G>A	c.499C>T	p.Q167*	0.74	0.93	I
21	64	O	HGSOC	I	S-117	PS	O	95%	*TP53*	17:7574012	C>A	c.1015G>T	p.E339*	0.70	0.70	I
22	65	O	HGSOC	III	S-128	IDS	O	95%	*TP53*	17:7579521	C>A	c.166G>T	p.E56*	0.68	0.67	I
23	54	O	HGSOC	III	S-138	IDS	OM	95%	*TP53*	17:7577569	A>C	c.712T>G	p.C238G	0.79	0.79	I
24	63	FT	HGSOC	III	S-146	PS	O	60%	*TP53*	17:7577559	G>T	c.722C>A	p.S241Y	0.62	0.62	I
25	61	PP	HGSOC	III	S-162	IDS	OM	40%	*TP53*	17:7578404	A>T	c.526T>A	p.C176S	0.11	0.19	I
26	72	PP	HGSOC	III	S-171	IDS	OM	50%	*TP53*	17:7578265	A>T	c.584T>A	p.I195N	0.71	0.70	I
27	68	O	HGSOC	III	S-179	IDS	O	90%	*TP53*	17:7578262	C>G	c.587G>C	p.R196P	0.83	0.86	I
28	46	O	HGSOC	III	S-109	IDS	O	50%	*TP53*	17:7577548	C>T	c.733G>A	p.G245S	0.40	0.39	I
29	82	O	HGSOC	III	S-118	IDS	OM	80%	*TP53*	17:7577568	C>G	c.713G>C	p.C238S	0.74	0.75	I
30	69	FT	HGSOC	III	S-130	PS	O	80%	*TP53*	17:7579389	G>A	c.298C>T	p.Q100*	0.75	0.79	I
31	64	O	HGSOC	III	S-172	IDS	OM	70%	*TP53*	17:7578406	C>T	c.524G>A	p.R175H	0.48	0.42	I
32	71	O	HGSOC	III	S-101	IDS	P	90%	*TP53*	17:7578370	C>T	c.559+1G>A	p.?	0.95	0.94	I
33	73	O	HGSOC	III	S-144	IDS	OM	70%	*TP53*	17:7577557	A>C	c.724T>G	p.C242G	0.44	0.47	I
34	54	O	HGSOC	IV	S-165	IDS	O	95%	*TP53*	17:7578527	A>G	c.403T>C	p.C135R	0.47	0.46	I
					S-135	B	OM	80%	*TP53*	17:7578527	A>G	c.403T>C	p.C135R	0.57	0.60	I
35	55	PP	S/END	III	S-106	PS	O	90%	*TP53*	17:7574021	C>A	c.1006G>T	p.E336*	0.96	0.96	I
					S-140			90%	*TP53*	17:7574021	C>A	c.1006G>T	p.E336*	0.96	0.96	I
36	52	O	HGSOC	III	S-111	PS	O	95%	*TP53*	17:7578502	A>T	c.428T>A	p.V143E	0.45	0.44	I
								95%	*TP53*	17:7579642	C>T	Intron	Intron	0.22	0.28	M
37	45	O	END	II	S-143	PS	O	95%	*TP53*	17:7577538	C>T	c.743G>A	p.R248Q	0.75	0.77	I
								95%	*PTEN*	10:89720778	A>G	c.929A>G	p.D310G	0.59	0.52	I
38	55	O	END	IV	S-122	IDS	O	80%	*PTEN*	10:89692791	A>G	c.275A>G	p.D92G	0.19	0.18	M
								80%	*PTEN*	10:89685287	A>G	c.182A>G	p.H61R	0.17	0.17	I
39	54	O	HGSOC	III	S-119	PS	OM	80%								U
40	71	O	HGSOC	III	S-133	IDS	OM	80%								Indel
41	66	O	HGSOC	III	S-169	IDS	OM	50%								Indel
42	68	FT	S/CC	III	S-147	IDS	O	90%								Indel
43	49	FT	HGSOC	III	S-176	PS	O	95%							FAIL	
44	73	PP	HGSOC	IV	S-177	PS	OM	50%							FAIL	
45	44	O	HGSOC	III	S-178	IDS	OM	30%							FAIL	

EX1003.027-.028.

144. A POSA would have understood that Table S3 identified 40 single-base substitutions (*i.e.*, SNVs), identified by the "I" in the last column titled "Identified

by Sanger sequencing," in the ovarian tumor sample. A POSA would also have understood that Table S3 shows that tumor-specific SNVs were identified via sequencing a tumor sample. A POSA would have also understood that such tumor-specific SNVs could be identified in the same manner by performing whole exome sequencing or whole genome sequencing. This is because the whole exome sequences and whole genome sequences include the SNVs.

145. As set forth above, Forshew discloses performing whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations.

c. [1.b]: performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume; and

146. Forshew discloses to a POSA performing targeted multiplex amplification to amplify 10 to 500 target loci encompassing different tumor-specific SNV mutations from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume.

147. Forshew discloses identifying ten different tumor-specific SNV mutations in a tumor sample by performing whole genome sequencing:

NEOGENOMICS EX.1002.074

> 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).***

EX1003.010 (emphasis added).

148. Table S7 includes the affected gene, genomic location of the amplicon, genomic location of mutation, reference allele, and mutant allele for the ten mutations and corresponding amplicons, as shown below:

NEOGENOMICS EX.1002.075

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

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

EX1003.033.

149. As I explain above, the mutations shown above are SNVs that arise from different genes and genomic locations. *Id.*; *see supra* Section IX.B.1.b.

150. Next, Forshew discloses performing targeted multiplex amplification, amplifying the ten different tumor-specific SNV mutations in cfDNA from a plasma sample:

> 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. ***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)***. The patient was treated with single-agent epirubicin (gray-shaded region). After 4 months off treatment, a CT scan showed progressive disease and the patient commenced further treatment with paclitaxel chemotherapy. The 10 mutations followed a common pattern of sharp decline in AF upon onset of therapy and an increase in AF upon disease progression after termination of therapy (Fig. 4D).

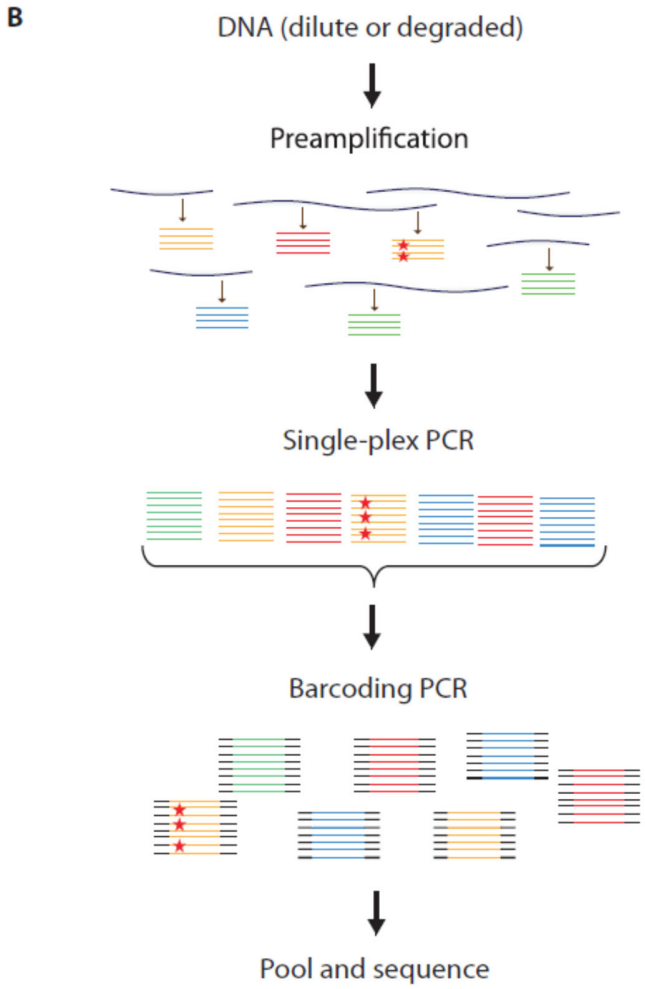
EX1003.010 (emphasis added); *id.* at .004 ("We therefore applied a two-step amplification process: a limited-cycle preamplification step where all primer sets were used together to capture the starting molecules present in the template, followed by individual amplification to purify and select for intended targets. (Fig. 1B) (Supplementary Methods)."); *id.* at .016 ("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."). Specifically, Forshew discloses performing preamplification reactions in volumes with "each forward and reverse target-specific primer" and "[r]eactions were subjected to 15 cycles of amplification." *Id*. ("Preamplification for TAm-Seq").

Figure 1(B) depicts 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." *Id.* (Fig. (1B) legend).

151. Patent Owner's expert witness in the district court case in North Carolina, Dr. Metzker, has confirmed that Forshew discloses the use of multiplex amplification in its preamplification step. EX1052 at 65:15-18 ("Q. And do you agree that the Forshew (2012) paper that does disclose the use of a multiplex amplification; correct? A. Forshew (2012) does describe multiplex PCR."), 66:19-24 ("Q. You do agree, though, that the Forshew (2012) publication we've been looking at, that it does use multiplex targeted application [sic]; correct? A. In the preamplification step, it does use multiplex PCR."), 39:19-23 ("Q. So you understand that the preamplification step that's described in Forshew (2012) is a reaction where all the targets are amplified together in the same tube? A. That's my understanding."). As I detail here, I agree with Dr. Metzker's testimony where he admits Forshew discloses multiplex amplification, that is, amplification of multiple targets together in the same reaction volume.

152. Forshew depicts its workflow graphically in Figure 1(B), reproduced below, beginning with the "initial preamplification step…using a pool of target-specific primer pairs" with the "diagram show[ing] DNA molecules that carry mutations (red stars) being amplified alongside wild-type molecules."

EX1003.003, Fig. 1(B) legend. Figure 1(B) then depicts the second step, a single-plex PCR, in which "[r]egions of interest" are "amplified in individual (single-plex) PCR, thus excluding nonspecific products." *Id.* Finally, Figure 1(B) depicts attaching "sequence adaptors and sample specific barcodes…to the harvested amplicons in a further PCR" before the products of the further PCR are pooled and sequenced. *Id.*



EX1003.003, Fig. 1(B).

153. With respect to cfDNA, Forshew discloses that "[s]erial 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." EX1003.010. Forshew similarly discloses that "[p]lasma of cancer patients contains cell-free tumor DNA that carries information on tumor mutations and tumor burden" and focuses on using TAm-Seq to monitor cfDNA. EX1003 at Abstract. A POSA would have understood that the use of DNA isolated from plasma teaches the use of cfDNA from plasma.

154. Forshew discloses amplicons with lengths of "<120 bp to cover each mutation." EX1003.033. Forshew also discloses amplicons with lengths of more than 50 bases in the listed primers defining amplicons (Table S1) and the listed genomic location of each amplicon with a start location and stop location (S7). *Id.* at .023-.024; *id.* at .033. These amplicons have lengths that fall within the recited range of 50-150 bases.

155. Forshew also discloses performing TAm-Seq with larger numbers of amplicons, which teaches that the degree of multiplex may be greater than ten. Specifically, Forshew discloses performing TAm-Seq with 48 sequence-specific primer pairs to amplify 5995 bases of genomic sequence, which cover the coding regions (*i.e.*, exons and exon junctions) of *TP53* and *PTEN*, as well as selected

regions in *EGFR*, *BRAF*, *KRAS*, and *PIK3CA* (Table S1) by the overlap of short amplicons (Fig. 1A). EX1003.004. Patent Owner's expert in the related case, Dr. Metzker, also testified that Forshew discloses using more than 20 pairs of target-specific primers in a multiplex reaction:

> Q. Now, let's go to Table S1.
>
> A. Yes, I'm there.
>
> Q. This is listing the target-specific primers that were used in that preamplification reaction?
>
> A. That's correct.
>
> Q. And there's more than 20 targets that are listed there?
>
> A. Well, I haven't counted them. Looks like 20.
>
> Q. There's more than 20; right? Because the table continues on the next page; right?
>
> A. You are correct.

EX1052 at 39:24-40:10. As detailed here, I agree with Dr. Metzker's testimony that Forshew discloses multiplex amplification using target-specific primers to amplify more than 20 targets in the same reaction.

156. Table S6 reports 23 point mutations (*e.g.*, SNVs) that were identified in plasma samples from ovarian cancer patients. EX1003.031-.032 (titled "Additional data for Table 2"); *id.* at .007 (Table 2 legend) (Table 2 reports "[m]utations identified by TAm-Seq in a set of 62 plasma samples from ovarian cancer patients."). Table S6 includes 23 unique SNVs as demonstrated by the 23

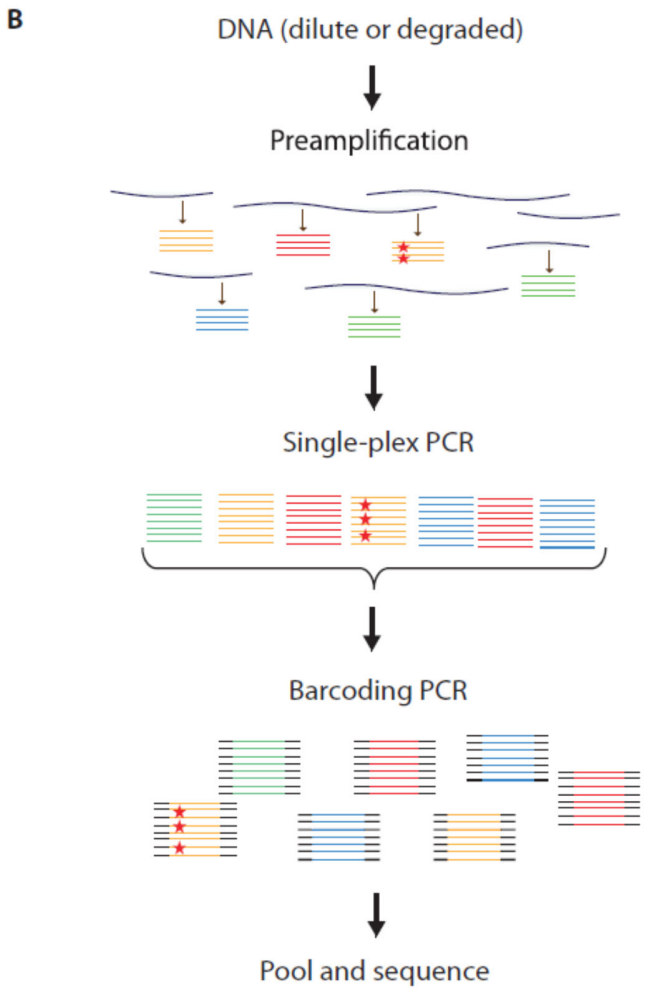
different "[l]ocation (build is hg19) and base change" for various samples. EX1003.031-.032. As explained above, TAm-Seq includes multiplex amplification with a combination of "each forward and reverse target-specific primer" in the same volume. *Id.* at .016.

157. As set forth above, Forshew would have taught or suggested to a POSA to perform targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume.

d. [1.c]: sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads, wherein the sequencing has a depth of read of at least 50,000 per target locus.

158. Forshew discloses the key components of this claim element, including sequencing to obtain sequence reads and detecting tumor-specific SNV mutations in cfDNA from the sequence reads.

159. Forshew discloses "sequencing...to obtain sequence reads." Figure 1(B) shows that the TAm-Seq method includes amplifying DNA by a targeted multiplex amplification step (*i.e.*, "Preamplification"), which is followed by "Single-plex PCR" and "Barcoding PCR" steps:



EX1003.003 at Fig. 1(B). Next, the amplified DNA is pooled and sequenced. *Id.*; *see also* EX1003.004 ("We performed 100-base single-end sequencing (GAIIx sequencer, Illumina), with an additional 10 cycles using the barcode sequencing primer, generating ~30 million reads per lane."); *see also id.* at .010 ("we demonstrate noninvasive identification of mutant alleles in plasma, at AFs as low as 2%, by targeted deep sequencing of circulating DNA"). Forshew also discloses "detecting one or more of the tumor-specific SNV mutations present in the cell-free

DNA from the sequence reads." After the DNA is sequenced, the resulting sequence data is analyzed to detect tumor-specific SNV mutations. Forshew teaches using a bioinformatics pipeline for this purpose. *See* EX1003.018 ("Analysis of Sequencing Data"); *see also id.* ("Mutation detection"). Forshew discloses identifying tumor-specific SNV mutations in plasma samples from seven ovarian cancer patients when the patients presented with relapse disease, as shown below:

Table 1. Mutations identified by TAm-Seq in plasma samples from seven ovarian cancer patients. TAm-Seq was used to sequence DNA extracted from plasma of subjects with HGSOC (stage III/IV at diagnosis). Plasma was collected when patients presented with relapse disease, before initiation of chemotherapy. For patient 46, DNA from a formalin-fixed, paraffin-embedded (FFPE) sample was not included in the TAm-Seq set and the mutation was validated in FFPE by Sanger sequencing. CA125 was measured at time of plasma collection. Mean depth of coverage at the mutation locus in the TAm-Seq data was averaged over the repeats (RMS deviation = 850). AF, allele frequency; N, no; Y, yes.

Patient ID	Age at diagnosis	Time elapsed since surgery (months); number of previous lines of chemotherapy	CA125 (U/ml)	Plasma per amplification reaction (µl)	Gene	Mutation and base change (genome build hg19)		Protein change	Detected in FFPE	Mean depth (sequencing reads)	Mean AF using TAm-Seq	Mean AF using digital PCR
8	60	13; 1	2122	50	*TP53*	17:7577120	C>T	p.R273H	Y	5000	0.09	0.10
12	62	27; 3	365	50	*TP53*	17:7577579	G>T	p.Y234*	Y	5000	0.10	0.08
14	58	50; 3	260	120	*TP53*	17:7578212	G>A	p.R213*	Y	5800	0.15	0.12
25	61	9; 1	944	110	*TP53*	17:7578404	A>T	p.C176S	Y	4800	0.04	0.08
27†	68	15; 1	1051	90	*TP53*	17:7578262	C>G	p.R196P	Y	7700	0.06	0.14
					EGFR	7:55259437	G>A	p.R832H	N	5700	0.06	0.05
31	64	12; 1	313	30	*TP53*	17:7578406	C>T	p.R175H	Y	4500	0.44	0.56
46	56	30; 2	1509	30	*TP53*	17:7578406	C>T	p.R175H	Y	4200	0.23	0.30

*Indicates stop codon. †Both a *TP53* and an *EGFR* mutation were identified in this sample (Fig. 4A).

EX1003 at Table 1, Table 1 Legend; *see also id.* at .016-.017; *id.* at .033 at Table S7 ("subsequent data analysis was performed using the CASAVA 1.8 analysis pipeline (Illumina) to identify somatic mutations."); *id.* at Abstract.

160. This claim limitation also requires that the sequencing "has a depth of read of at least 50,000 per target locus." As described below, to the extent Forshew

is found to not disclose these elements, it is my opinion that a POSA would have been motivated to modify Forshew to achieve the claimed invention and would have had a reasonable expectation of success.

(i) A POSA Would Have Increased Sequencing Depth To Increase Sensitivity

161. This claim limitation requires that the "sequencing has a depth of read of at least 50,000 per target locus."

162. A POSA would have understood that sequencing "depth of read," as described in the '454 patent, is a measure of the number of times a particular sequence is read or number of sequence reads that map to a given genetic locus. This is clear from how the '454 patent describes "depth of read." *See* EX1001 at 100:9-15 ("Depth of read can be expressed in variety of different ways,…for example in a highly parallel DNA sequencer such as an Illumina HISEQ, which, e.g., produces a sequence of 1 million clones, the sequencing of one locus 3,000 times results in a depth of read of 3,000 reads at that locus.").

163. A POSA would have understood that Forshew discloses sequencing depths of up to 15,400 for identifying tumor-specific SNV mutations in ovarian tumor samples. EX1003.004; *id.* at .006 at Table 1. Specifically, Table 1 discloses the mean sequencing depth for numerous mutations, including a mean sequencing depth of 7,700 for one mutation. *Id.* at .006 at Table 1. With respect

to this testing, Forshew discloses "amplif[ying] DNA from each sample in duplicate, tagging each replicate with a different barcode," followed by sequencing. *Id.* at .004. Thus, the sequencing data was obtained in duplicate for each sample. A POSA would have understood that the mean sequencing depth of 7,700 for duplicate samples is a total sequencing depth of 15,400 (*i.e.*, twice 7,700).

164. To the extent Patent Owner argues or it is found that Forshew does not disclose a sequencing depth of at least 50,000, such a sequencing depth would have been obvious to a POSA in view of Forshew alone. As noted above, a POSA would have understood that Forshew already teaches sequencing depths greater than 15,400. A sequencing depth of 50,000 is nothing more than an obvious variation on 15,400 because 50,000 is also on the order of tens of thousands.

165. As I explain further below, a POSA armed with knowledge of Forshew would have been motivated to increase sensitivity by sequencing more molecules and reaching a read depth of at least 50,000 because sequencing more molecules in a given region of the genome allows for more frequent sampling of the sequence associated with that region, increasing the sensitivity in detecting SNVs and would have had a reasonable expectation of success. This is clear from Forshew, the prior art, numerous admissions from Patent Owner and its experts, and even the '454 patent itself.

NEOGENOMICS EX.1002.086

166. Forshew discloses that it "demonstrate[d] noninvasive identification of mutant alleles in plasma, at AFs as low as 2%." EX1003.010. A "current limitation of Tam-Seq" in Forshew, however, was its "detection limit compared to assays that target individual loci," which were able "to detect two to three orders of magnitude lower frequencies." *Id.* at .011; *see also id.* at .010 ("[e]nhanced detection down to 1 mutant allele in 10,000 or more wild-type alleles [0.01% SNP frequency] can be obtained using a variety of methods."). Forshew teaches a POSA how to overcome this and achieve enhanced mutation detection.

167. Specifically, Forshew teaches that sequencing at greater depths of read was desirable to increase the sensitivity of detection, as it was intended to monitor ctDNA associated with the recurrence of cancer, which can occur at low amounts. EX1003 at Abstract; *id.* at .011. Forshew expressly teaches that "[***h]igher read depth*** or fidelity, ***additional replicates***, or improved algorithms could allow for ***enhanced mutation detection without change to protocols***." *Id.* at .011 (emphasis added). While this passage refers expressly to "read depth," a POSA would have understood that using "additional replicates" is also using "higher read depth" because depth of read is just a measure of the number of times a particular sequence is read or sequence reads that map to a given genetic locus, and one can increase the number of such reads by using "additional replicates." *Id.*

168. A POSA would have understood that it was routine to increase sequencing read depth to obtain greater sensitivity and would have had a reasonable expectation of success in doing so. This is so because such an approach was well-known in the art. For example, Patent Application US 2010/0261189 A1 (EX1057) ("Bentley"), which published October 14, 2010, expressly teaches that the higher the number of reads, the lower the limit for the minimum frequency of detectable SNVs. EX1057 ¶ 85. Bentley also expresses what a POSA would have understood about the relationship between "Number of Reads" and the "Minimum frequency of SNP in population detectable with 95% confidence…[and] 99% confidence," as shown below:

TABLE 2

SNP Classes	Number of Reads	Minimum frequency of SNP in population detectable with 95% confidence	Minimum frequency of SNP in population detectable with 99% confidence
1	200000	0.002%	0.003%
2	100000	0.005%	0.007%
5	40000	0.014%	0.018%
10	20000	0.028%	0.037%
50	4000	0.14%	0.18%
100	2000	0.28%	0.37%
200	1000	0.55%	0.74%
500	400	1.39%	1.85%
1000	200	2.76%	3.64%

EX1057 ¶ 88 (Table 2). Specifically, Bentley reflects a POSA's understanding that a sequencing depth of 40,000 would, with 95-99% confidence, successfully detect SNPs between a frequency of 0.014% and 0.018%. *Id*. Bentley also reflects a POSA's understanding that a sequencing depth of 100,000 would, with 95-99% confidence, successfully detect SNPs between a frequency of 0.005 and 0.007%. *Id*. To reach Forshew's teaching of enhanced detection for alleles with 0.01% frequency, a POSA would have been motivated to use and would have reasonably expected success with a sequencing depth of up to 100,000 based on the POSA's understanding as reflected in Bentley. *Id*. Bentley is consistent with a POSA's understanding that sequencing depth is routinely adjusted to achieve the required sensitivity. Moreover, other prior art similarly teaches that there is a relationship between increasing sequencing reads and improving sensitivity and that the detection limit is determined by sequencing read depth and error rate. *See, e.g.*, EX1105.001; EX1005 at Fig. 2 (showing correlation between increasing reads and increasing sensitivity); EX1108.011 ("Accuracy in NGS is achieved by sequencing a given region multiple times…with each sequence contributing to 'coverage' depth. Through this process, a 'consensus' sequence is derived.").

169. The use of sequencing depths greater than 50,000 was not just disclosed in Bentley, but was well-known in the art. For example, Narayan et al., "Ultrasensitive Measurement of Hotspot Mutations in Tumor DNA in Blood Using

Error-Suppressed Multiplexed Deep Sequencing," Cancer Res., 2012, Vol. 72(14) (EX1086) ("Narayan"), published July 15, 2012, expressly discloses a sequencing depth of 108,467 reads per locus. EX1086.003 ("We obtained a median depth of 108,467 read pairs per mutation site per sample after filtering and demultiplexing a total of 86,359,980 raw sequences generated on a single lane of an Illumina HiSeq 2000 flow cell"); *see also* EX1090 ¶ 104; *see also* EX1101.012-.013 (EX1102, EX1132). The sequencing depth in Narayan was obtained by performing NGS of tumor-specific mutations in cfDNA from lung cancer patients. EX1086.003; *see also* EX1090 ¶ 104. Narayan explains that "[b]y designing PCR primers that flank very short regions (<50 bp) surrounding these mutation hotspots, we could ensure adequate amplification of highly fragmented plasma DNA and achieve greater sequence depth." EX1086.003 (emphasis added); *see also* EX1090 ¶ 104 (disclosing "median depth of read of 108,467 read pairs…per mutation site per sample after filtering and demultiplexing" derived from plasma samples); *see also* EX1101.012 -.013 (EX1102, EX 1132) (sequencing tumor tissue to depths up to 3 million, with over 50 variants validated with sequencing depths over 50,000).

170. Patent Owner's positions in prior cases and proceedings further confirm it was well-known in the art that increasing sequencing depth improves sensitivity. For example, Patent Owner has asserted that "[i]t was well-known by 2009 that the number of molecules analyzed is one component of sensitivity, and that higher

sensitivity could be achieved by sequencing more molecules." EX1088.032-.033; *see also id.* at .033 ("it was well-known by 2009 that by sequencing more molecules, sensitivity will necessarily increase.").

171. Similarly, one of Patent Owner's experts, Dr. John Quackenbush, opined that:

> [S]equencing to even greater depth when required by an experiment also would have been routine, as it would require only running additional sequencing assays: "it is very easy to increase the coverage or sequence depth, if you later decide you need more data. Provided you still have your original sample, you can just sequence more, and combine the sequencing output from different flow cells."

EX1095 ¶ 65.

172. In another case, Dr. Quackenbush opined that "POSAs prior to November 6, 2009 were well aware that sequencing more molecules, or increasing sequencing depth, improves sensitivity" and that "the detection limit is determined by sequencing error rate and sequencing read depth (i.e., how many molecules are sequenced), reflecting the fact that there was a well-known correlation between the number of molecules sequenced and sensitivity." EX1094 ¶ 119 (citing EX1108).

173. Dr. Quackenbush also testified that to get higher sensitivity, you simply sequence more molecules and this was well-known:

> Q. Does the written description state anything about how a person of skill in the art can increase the sensitivity of high-throughput sequencing?

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> A. So ***they do provide a description of how to increase sensitivity***, which is simply to obtain more sequence, right, which is well-known with any kind of measurement. ***If you want to make a better measurement, you measure more.***
>
> Q. Can you point to an example of that in the written description?
>
> A. Sure. It's right here. It's called out. It just says, ***higher sensitivity can be achieved simply by sequencing more molecules***. That means use more channels. And when they refer to channels, the Illumina sequencer had something that looked like a microscope slide added onto individual channels. Simply showed you load one, you load two, you load three, you load four.
>
> Q. And is there anything nonconventional about achieving higher sensitivity by sequencing more molecules?
>
> A. No. I mean, this is standard practice in many fields. To make a better measurement, you make more measurements. ***To get higher sensitivity for detecting molecules, you simply sequence more molecules***.

EX1084 at 98:10-99:6 (emphasis added). I agree with the foregoing positions from Patent Owner in its prior litigation.

174. Dr. Metzker, Patent Owner's expert in parallel district court litigation, has also testified to the Patent Trial and Appeal Board ("the Board") that it would have been obvious to increase sequence coverage (*i.e.*, sequencing depth) to improve the accuracy of NGS platforms. EX1082 ¶¶ 91, 211-215. Specifically, Dr. Metzker opined that in the 2010 timeframe, to "increase the accuracy of the Illumina GA sequencer, it would have been obvious in my opinion to target regions of a genome with high sequence coverage," where "sequence coverage" has the same

meaning as "sequence depth"—the number of unique sequence reads. *Id.* ¶ 214. Dr. Metzker relied on prior art for teaching "***[a]t high sequence coverage all NGS platforms have excellent variant calling accuracy*** (>95%) as assessed by the detection of known SNP variants." *Id.* ¶ 213 (citing EX1042). I agree with Dr. Metzker's opinions to this effect.

175. In my opinion, the '454 patent itself provides further confirmation that a sequencing depth of 50,000 would have been obvious. Specifically, the '454 patent does not identify any particular sequencing depth as inventive or special. Instead, it only refers to a sequencing depth of 50,000 once, in a long list of possible sequencing depths spanning 100 to 500,000 reads. EX1001 at 68:33-39 ("[i]n certain embodiments, a depth of read of greater than 100, 250, 500, 1,000, 2000, 2500, 5000, 10,000, 20,000, 25,000, 50,000, or 100,000 on the low end of the range and 2000, 2500, 5,000, 7,500, 10,000, 25,000, 50,000, 100,000, 250,000 or 500,000 reads on the high end, is attained in the sequencing run for each single nucleotide variant position in the set of single nucleotide variant positions."). Put another way, if a sequencing depth of 50,000 were truly inventive or novel, the specification surely would have included some disclosure to this effect. To a POSA, the specification's broad disclosure of a broad range of sequencing depths without any specific identification of 50,000 is a further indicator of the fact that sequencing depth is routinely adjusted for the desired sensitivity.

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176. I understand Patent Owner has argued in parallel district court litigation, and may contend in this proceeding, that it would not have been obvious to increase sequencing depth as taught by Forshew because increasing sequence depth in connection with the TAm-Seq process would have presented challenges that would have dissuaded a POSA. EX1054.011-.012; EX1081 ¶¶ 81-118. Specifically, Patent Owner has argued that "[s]equencing each SNV locus 50,000 times or more…would not reasonably be expected to identify rare variants in low-abundance ctDNA above the background 'noise' from sequencing-related error rates." EX1054.012. Patent Owner's expert, Dr. Metzker, likewise opined that a POSA "would not have understood the claimed ultra-deep > 50,000 sequencing depth to be something that would remediate the admitted limited sensitivity described in Forshew (2012) and elsewhere." EX1081 ¶ 82. Further, Dr. Metzker opined a POSA "therefore would not have expected ultra deep sequencing to a depth of read of at least 50,000 per target locus to enable the Forshew (2012) method to analyze rare mutations in cfDNA." *Id.* ¶ 83. Dr. Metzker further opined that "[p]rior to 2015 there were many challenges in using next-generation sequencing" and "Forshew (2012)'s sample library preparation and sequencing workflow exceed the technique's ability to detect rare genetic DNA variants…the rare variants are lost in the 'noise' associated with the abundant errors found in the sequence read data." *Id.* ¶¶ 93-94. I disagree with all of this.

177. As an initial matter, I note that Dr. Metzker has repeatedly admitted that a POSA would have a high level of skill, which is incompatible with the image of an incompetent artisan he seeks to paint. EX1055 at 15:23-16:20, 18:24-19:9, 21:24-22:8, 26:2-14, 26:24-27:17, 37:12-24, 38:2-11, 102:25-103:7. Dr. Metzker has opined that it would have been "obvious" to increase sequencing depth to enhance accuracy. EX1082 ¶ 214. Dr. Metzker has also opined that as of 2009, the literature taught that at "high sequence coverage ***all*** NGS platforms have ***excellent variant*** calling ***accuracy*** (>95%) as assessed by the detection of known SNP variants." *See* EX1082 ¶ 213 (citing EX1042) (emphases added). In parallel district court litigation, Dr. Metzker has similarly opined about the use of enzymes with built-in "proofreading" capabilities that would overcome PCR errors that would allegedly cause problems for sequencing. *See* EX1081 ¶ 304. A POSA would have appreciated that both higher sequencing depth and higher fidelity improve sensitivity, and that increased fidelity means lower background error rates. EX1003.011; *see also* EX1081 ¶ 97 ("[S]killed artisans understood that although increasing read depth could potentially increase sensitivity, it would not increase the sensitivity beyond the limit of detection based on the error rate."); *see also* EX1081 ¶ 102 ("ultra-deep sequencing can" find a "needle in the haystack").

178. I understand Patent Owner has nonetheless argued that increasing sequencing depth as shown in Bentley would have been discounted by a POSA

because a POSA would have believed that Bentley failed to account for errors introduced by polymerase or other sequencing errors, such as it may contend are present in Forshew. I disagree with this. Such polymerase and sequencing errors can be either systematic or random errors. "Systematic error manifests as many individual base-call errors from separate sequence reads occurring at the same genomic position." EX1128.002. A POSA would have been aware of routine methods for correcting for systematic errors. *See, e.g.*, *id.* at Abstract (disclosing development of computer program to identify and correct for systematic errors); EX1127 at Abstract (discussing solutions to systematic bias in next generation sequencing data); EX1129 at Abstract ("Many techniques for error correction of sequencing data from next-gen platforms have been developed in the recent years….In this article, we provide a comprehensive review of many error-correction methods."). Systematic errors, thus, would not impact the analysis because a POSA would know how to accommodate these errors and account for them. For random errors, the same error in reading a particular base pair does not generally occur repeatedly. Rather, different errors occur at random base pairs. Such random errors are precisely the type of errors that a POSA would understand can be overcome by sequencing more molecules and ultimately arrive at a more accurate sequence determination. Put another way, a POSA, with an understanding of the nature of polymerase and sequencing errors, would have understood that the way to

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address those errors and increase sensitivity would be to increase sequencing depth. A POSA would understand that this is precisely why Forshew disclosed this in the first place.

179. Further, a POSA would have understood that between 2012 (when Forshew was published) and 2015, which is the priority date of the patent, the fidelity and cost of sequencing technology underwent dramatic improvements, such that a POSA would have been even more motivated to increase sequencing depth. *See generally* EX1054.011-.012; EX1081 ¶¶ 81-118.

180. For example, while a high error rate may, at a point, limit the beneficial effect of increasing sequencing depth, using higher fidelity polymerases, such as proofreading polymerases, reduces the error rate and allows benefit from greater sequencing depth. *See* EX1081 ¶ 291 ("One of ordinary skill in the art would have readily recognized that an additional PCR could be performed using a proofreading polymerase (as opposed to Taq polymerase), such that there is no substantial increase in error."); EX1055 at 37:12-24. Thus, a POSA would have understood that the sensitivity of detection could be improved by both increasing the sequencing depth ***and*** fidelity of sequencing and that the two would go hand-in-hand. EX1003.011.

181. I note that the foregoing is consistent with Dr. Metzker's testimony in the parallel district court litigation. *See* EX1081 ¶ 291 ("One of ordinary skill in

the art would have readily recognized that an additional PCR could be performed using a proofreading polymerase (as opposed to Taq polymerase), such that there is no substantial increase in error."); EX1055 at 37:12-24 (a POSA "would have understood how to use proofreading polymerases.").

182. Notably, a POSA would have understood Forshew itself to expressly teach increasing fidelity of the amplification process and appreciated well-known improvements that decreased the error rates in generating amplicons. Specifically, Forshew uses FastStart High Fidelity Enzyme Blend (Roche) (EX1003.016), which a POSA would have known relies on a *Taq* polymerase, but with improved accuracy compared to normal *Taq* polymerase. EX1120 ("The FastStart® High Fidelity PCR System – an optimized enzyme blend for hot start PCR – combines all the benefits of FastStart Taq DNA Polymerase with fourfold higher accuracy and the ability to amplify fragments up to 5kb."); EX1122; EX1098.002 (reporting an accuracy of 6 times higher than *Taq* polymerase).

183. Other prior art is consistent with Forshew and discloses the use of improved polymerases. For example, Bashashati uses Phusion DNA polymerase (NEB, Ipswich, MA, USA). EX1004.020-.021. As another example, Weaver uses Q5 HotStart High-Fidelity Polymerase (as well as Phusion High-Fidelity DNA Polymerase (NEB)). EX1110.009. A POSA would have recognized these polymerases as having greater fidelity than *Taq* polymerase. *See, e.g.*,

EX1089.002 (reporting Phusion® having fidelity greater than 50x *Taq* and Q5™ greater than 100x *Taq*); EX1080.004 (comparing error rates of different PCR reactions, including *Taq*, *Pwo* (Roche), and Pfusion); EX1080.006; *see also* EX1121.001 (reporting that Q5 DNA polymerase available from New England Biolabs was reported to have "the highest fidelity amplification available (>100 times higher than Taq)" and "result[] in ultra-low error rates."). A POSA would have reasonably expected success in using these available high fidelity DNA polymerases to improve the fidelity of amplification and decrease the error rate in generating amplicons.

184. As to increasing the fidelity of the sequencing process itself, a POSA would have appreciated well-known improvements in sequencing methods and reagents and would have adopted those with increased fidelity to improve sensitivity as Forshew expressly teaches. EX1003.011.

185. A POSA would have appreciated the significant advances made in sequencing technology in the time period between 2012 when Forshew was published and 2015, including as to the fidelity of sequencing. *See, e.g.*, EX1078.002 ("improvements in labeling chemistries and/or detection sensitivities have resulted in ever-increasing data output per sequencing run in NGS platforms"); EX1068.015 ("In 2010, the 1000 Genomes Project released its initial results from WGS [whole genome sequencing] of 179 individuals and targeted sequencing of 697

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individuals. As of 2015, the genomes of 2,504 people from 26 different populations have been reconstructed, providing an unparalleled insight into human variation on a population level, and projects to sequence even larger sets of people are nearing completion or are underway").

186. In the parallel district court litigation, Dr. Metzker opined that "relatively high-fidelity sequencing platforms were known to introduce sequencing errors at a rate of $\geq$ 0.1%." EX1081 ¶ 94 (citing EX1067 at Table 3). Dr. Metzker, however, ignored that the Glenn reference he cites discusses sequencers with error rates as low as 0.01%, which would be sufficient for detecting an SNV at a frequency of 0.01%. *Id.*; EX1067 at Table 3. Indeed, Dr. Metzker admitted that a POSA would have been aware of Glenn and its disclosures. EX1055 at 102:25-103:7; *see also id.* at 101:25-102:23 (admitting that a POSA would have been aware of error rates of commercial sequencing platforms). I agree with Dr. Metzker that a POSA would have been aware of Glenn and the error rates of commercial sequencing platforms.

187. In the parallel district court litigation, Dr. Metzker cites an article by Schmitt et al., which purports to disclose "that base-specific mutagenic DNA damage followed by polymerase misincorporation against such damaged base contributes the error rate of the sequencing process." EX1081 ¶ 95 (citing EX1100). Dr. Metzker, however, ignores that Schmitt discloses Duplex

Sequencing, which has "a theoretical background error rate of less than one artifactual [sic] mutation per billion nucleotides sequenced." EX1100 at Abstract. Again, Dr. Metzker admitted that a POSA would have been aware of Schmitt's disclosures. EX1055 at 96:20-98:10. So, Dr. Metzker's own reference undercuts his conclusion—to the extent a POSA would have had any concern about the types of sequencing errors discussed in Schmitt, a POSA could have readily applied the disclosed Duplex Sequencing method to remediate those errors. EX1100 at Abstract. Dr. Metzker also cites an article by Kinde et al., which purports to disclose that "massively parallel sequencing (sometimes also referred to as "next generation sequencing" or "NGS") could not have been used generally to analyze rare variants because of high error rates associated with the sequencing workflow." EX1081 ¶ 96 (citing EX1074). Kinde, however, discloses the Safe-SeqS method, which "can substantially increase the sensitivity of massively parallel sequencing instruments for" identifying "mutations that are present in a small fraction of DNA templates." EX1074 at Abstract. Again, Dr. Metzker admitted that a POSA would have been aware of Kinde's disclosures. EX1055 at 104:16-105:7. And Kinde itself undercuts Dr. Metzker's conclusion—to the extent a POSA would have had any concern about the types of errors discussed in Kinde, a POSA could have readily applied the disclosed Safe-SeqS method to remediate those errors. EX1074 at Abstract.

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188. In addition to improvements in capabilities as to fidelity and sensitivity, the drastic decline in sequencing cost by 2015, including just the period between when the work in Forshew was performed (*i.e.*, prior to October 2011 when Forshew was submitted for publication) and 2015, would have motivated a POSA to further increase the sequencing depth in order to improve the ability to identify mutations with lower allelic frequencies. EX1119; EX1086.006 ("[S]equencer speeds have improved dramatically in just the past several months, and this trend is likely to continue. Thus, it does not seem that cost and time will remain significant impediments for much longer.").

(ii) A POSA Would Have Been Motivated To Modify Forshew To Omit Intermediate PCR Steps

189. To the extent the claims are deemed to require sequencing of the amplicons directly after the targeted multiplex amplification, a POSA would have understood that Forshew could also be modified so that the amplicons from the targeted multiplex amplification step would be sequenced directly without any intermediate PCR steps, omitting both the single-plex PCR and the PCR to introduce barcodes and sequencing adapters. Likewise, a skilled artisan would have been motivated to carry out this modification and would have had a reasonable expectation of succeeding.

190. As to omitting the single-plex PCR step, Forshew teaches that the preceding multiplex PCR was designed to avoid unwanted side products:

> The final concentration of each primer in the preamplification reaction was 50 nM, ***reducing the potential for interprimer interactions***, and 15 cycles of long-extension (4 min) polymerase chain reaction (PCR) were used to ***remain in the exponential phase of amplification***.

EX1003.004 (emphasis added). Likewise, Forshew explained that "[p]rimer designs were screened against the hg19 reference genome to prevent cross-product generation, and were screened to prevent primer-dimer interactions and formation of intramolecular secondary structure." EX1003.016. A POSA would have understood, accordingly, that the single-plex PCR performed in Forshew to improve purity was not necessary. Further, as Dr. Metzker confirmed, "[i]f the number of cycles remains within the exponential amplification phase, the relative abundance of mutations will not be diluted." EX1081 ¶ 290. In this regard, Forshew's multiplex PCR, which remained in the exponential phase, was designed in such a way that would preserve the relative abundance of mutations allowing direct sequencing of amplicons.

191. As to omitting the PCR step to introduce barcodes and sequencing adapters, a POSA would have understood that the sample barcodes and sequencing adapters could have easily been incorporated into the sequence-specific primers used

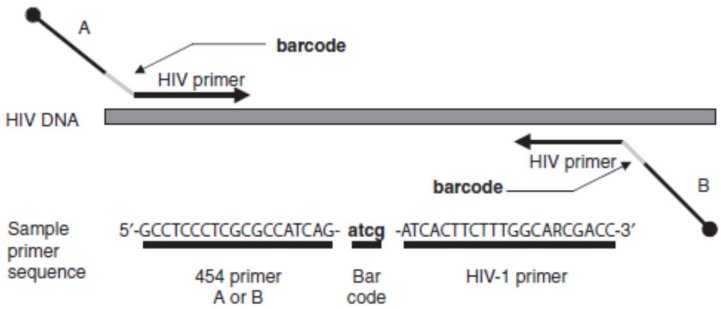
in the targeted multiplex amplification such that they would then be incorporated into the amplicons that result from the targeted multiplex amplification step. This would have then allowed the amplicons from the targeted multiplex amplification to be directly sequenced.

192. A POSA would have been motivated to modify Forshew in the foregoing manner because omitting the single-plex PCR step and performing targeted multiplex amplification using primer pairs having sample barcodes and sequencing adapters would simplify the overall process, requiring a single amplification step (as opposed to a separate amplification step to add the barcodes).

193. A POSA would have had a reasonable expectation of success in pursuing this approach for at least two reasons. First, as noted above, Forshew already teaches multiplex PCR conditions that would avoid unwanted side products and preserve relative abundance of mutations, such that the single-plex PCR that Forshew used to enhance purity would not be required. Second, Forshew discloses targeted multiplex amplification using target-specific primer pairs that include an additional sequence, namely, the "universal primer sequences (termed CS1 and CS2) appended at the 5'-end." EX1003.016.

194. This understanding that targeted multiplex amplification using a single amplification reaction step can be used to attach sequencing platform-specific adaptors and unique sample barcodes prior to sequencing is well-established in the

prior art. For example, Hoffmann et al., "DNA bar coding and pyrosequencing to identify rare HIV drug resistance mutations," Nucleic Acids Research, 2007, Vol. 35, No. 13 (EX1069), published June 2007, teaches that "[g]enomic DNA samples of interest are amplified using primers that include 5' extension providing binding sites for the 454A and B primers" that are required for sequencing. EX1069.003. This single amplification step, including the primers used, is illustrated in Figure 2:



Id. at Fig. 2;

> **Figure 2.** The DNA bar coding system. Primers are shown diagrammatically, illustrating the placement of the DNA bar code between the 454 primers (**A** and **B**) and the HIV-complementary sequences. An example of a primer sequence is shown at the bottom. DNA 50 ends in the primers are shown as the black balls.

Id. at Fig. 2, legend.

195. As set forth above, Forshew teaches or suggests to a POSA to sequence the amplicons to obtain sequence reads and to detect one or more of the tumor-specific SNV mutations present in the cell-free DNA, wherein the sequencing has a depth of read of at least 50,000 per target locus.

2. Claim 2: The method of claim 1, wherein the cell-free DNA comprises circulating tumor DNA.

196. Forshew discloses to a POSA the method of claim 1, wherein the cell-free DNA comprises circulating tumor DNA.

197. As discussed above with respect to claim 1, Forshew extracts, amplifies, and sequences cfDNA from plasma, detecting tumor-specific SNV mutations in the cfDNA. *See supra* Section IX.B.1. Forshew teaches that this cfDNA is circulating tumor DNA. EX1003 at Abstract ("Plasma of cancer patients contains ***cell-free tumor DNA*** that carries information on tumor mutations and tumor burden.") (emphasis added); *id.* at .005 ("For all seven patients, TP53 tumor mutations were identified in the circulating DNA at frequencies of 4% to 44% (Table 1).").

198. As set forth above, Forshew would have taught or suggested to a POSA to perform the method of claim 1, wherein the cell-free DNA comprises circulating tumor DNA.

3. Claim 3: The method of claim 1, wherein the SNV mutations comprise one or more clonal SNV mutations.

199. Forshew in view of the skill and knowledge of a POSA discloses and renders obvious the method of claim 1, wherein the SNV mutations comprise one or more clonal SNV mutations.

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200. A POSA would have understood clonal SNV mutations to be mutations present in the ancestral (original) clone of the tumor cells, as cancer cells are assumed to originate from monoclonal compositions. EX1005.001-.002 ("Although all malignant cells are thought to be derived from a common precursor cell, acquired genetic instability gives rise to subsequent generations expressing unique characteristics."); EX1004.002 ("cancers are assumed to originate from a monoclonal composition (the ancestral clone)."). Forshew discusses tracking "multiple mutations" that "can provide insight into clonal evolution and, at the same time, increases the robustness for tumor monitoring by compensating for effects of sampling noise or mutational drift." EX1003.011. Forshew also cites to prior work discussing "mutation profiles in different metastatic clones," which "can differ significantly from each other or from the parent primary tumor (12, 13)." *Id.* at .002.

201. One of the problems Forshew teaches, and which it attempts to solve, is that evolutionary "changes within the cancer can alter the mutational spectrum of the disease and its responsiveness to therapies, which may necessitate repeat biopsies." EX1003.002. A POSA would have understood this to refer to the challenge of monitoring mutations that occur as cancer progresses. Forshew thus teaches that tumors will provide clonal and subclonal DNA. *Id.* A POSA would have understood this at the time of the '454 patent not only because of Forshew, but

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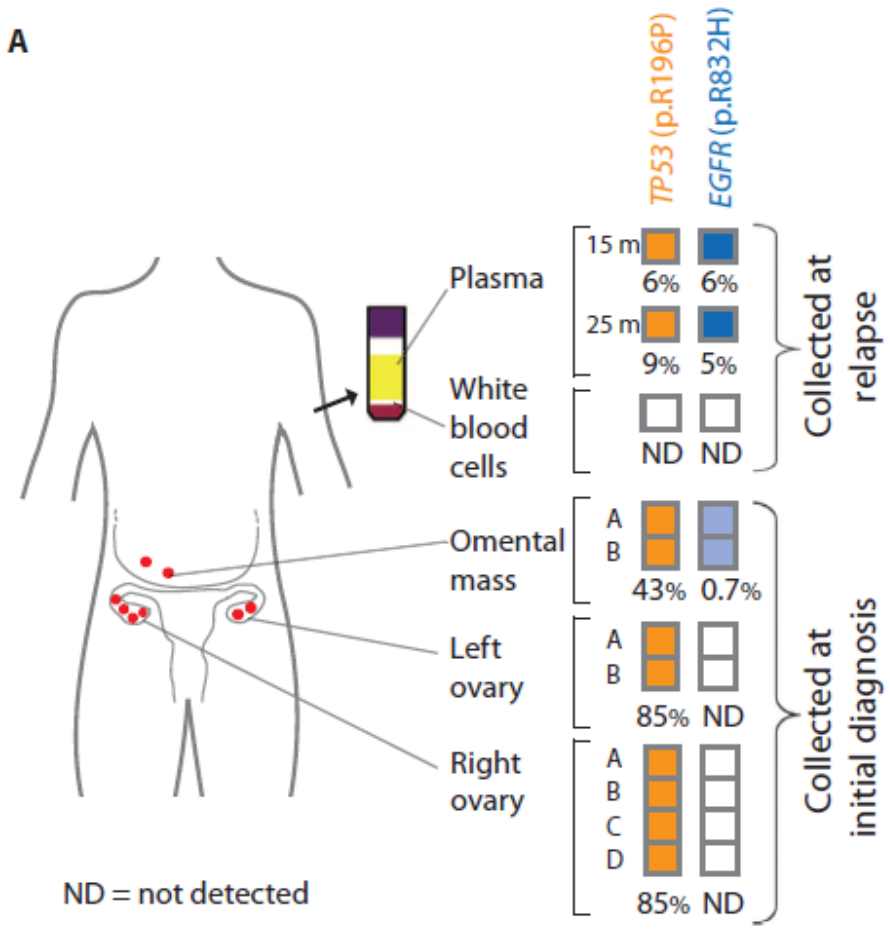
also from a plethora of prior art available at the time, including the above and, for example, Bashashati and Bai, which are both discussed in greater detail below.

202. Forshew discloses such a clonal SNV mutation. Specifically, tumor cells are identified as present in omental mass, left ovary, and right ovary, based on the presence of *TP53* mutation in all eight different regions of the tissues sampled.



EX1003 at Fig. 4(A). As each region in which tumor cells are identified establishes the presence of the *TP53* mutation, a POSA would have understood that SNV to be a clonal SNV mutation.

203. As set forth above, Forshew would have taught or suggested to a POSA to perform the method of claim 1, wherein the SNV mutations comprise one or more clonal SNV mutations.

4. Claim 4: The method of claim 1, wherein the SNV mutations comprise one or more subclonal SNV mutations.

204. Forshew in view of the skill and knowledge of a POSA renders obvious the method of claim 1, wherein the SNV mutations comprise one or more subclonal SNV mutations.

205. A POSA would have understood subclonal SNV mutations to be mutations arising during subsequent evolution of cancer cells, which may have distinct phenotypic properties. EX1005.001 ("monoclonal cancer cells gradually become heterogeneous owing to clonal evolution and genetic/epigenetic instability."); EX1004.002 ("cancers are assumed to originate from a monoclonal composition (the ancestral clone), with subsequent evolution leading to selective expansions of genomically distinct subclones [12] with distinct phenotypic properties [12-16]."). As discussed above with regard to claim 3, which requires identification of clonal SNV mutations, Forshew discloses that tracking "multiple mutations can provide insight into clonal evolution," EX1003.011, which a POSA would have understood to mean subclonal SNV mutations. A POSA would have understood this because cancers originate from a monoclonal composition, diverging

into genomically distinct subclones. *See, e.g.*, EX1004.002 ("Rooted in the clonal evolution model of tumour progression, cancers are assumed to originate from a monoclonal composition (the ancestral clone), with subsequent evolution leading to selective expansions of genomically distinct subclones [12] with distinct phenotypic properties [12–16].") (citing numerous articles dating to as early as 1976).

206. Forshew discloses such subclonal SNV mutations in the SNVs being detected in only a subset of regions in which cancer was detected. Specifically, tumor cells are identified as present in omental mass, left ovary, and right ovary, based on the presence of *TP53* mutation in all eight different regions of the tissues sampled, while a particular *EGFR* mutation is only present in two regions from the omental mass. EX1003 at Fig. 4(A) (reproduced above). The difference in the *EGFR* SNV between one tumor and another in the same patient would have shown a POSA that the tumor cells present in the patient had SNV mutations that included a subclonal SNV mutation.

207. Alternatively, Forshew renders this claim obvious because a POSA would have understood that cancerous tumors constantly mutate, and some of the mutations only occur in a subset of the tumor. As any of these SNVs, subclonal or not, can be called through whole genome sequencing of the tumor and healthy tissue, a POSA would understand that some of the SNVs called would be subclonal. Additionally, a POSA would have been motivated to monitor subclonal mutations

because, as discussed (*see supra* Section VI.F.), subclonal mutations, and clonal heterogeneity in general, have been linked to treatment resistance. Tracking of such subclonal mutations would benefit patients through better cancer detection, prognosis, and treatment. *See supra* Sections VI.E-F.

5. Claim 5: The method of claim 1, wherein the SNV mutations comprise one or more clonal SNV mutations and one or more subclonal SNV mutations.

208. Forshew in view of the skill and knowledge of a POSA renders obvious the method of claim 1, wherein the SNV mutations comprise one or more clonal SNV mutations and one or more subclonal SNV mutations.

209. As discussed above with regard to claims 3-4, Forshew teaches detecting both clonal and subclonal SNV mutations in circulating tumor DNA from plasma samples. EX1003 at Fig. 4(A); *id.* at .010-.011; *see supra* Sections IX.B.3-IX.B.4.

210. Alternatively, as discussed above with regard to claims 3 and 4, Forshew renders obvious detecting both clonal and subclonal SNV mutations in circulating tumor DNA from plasma samples. *See supra* Sections IX.B.3-IX.B.4.

6. Claim 6: The method of claim 1, wherein the tumor sample of the subject is a tumor tissue sample.

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211. Forshew in view of the skill and knowledge of a POSA renders obvious the method of claim 1, wherein the tumor sample of the subject is a tumor tissue sample.

212. As discussed above with regard to claim 1, which requires performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject, Forshew discloses and teaches using a tumor tissue sample. EX1003.010 ("Whole-genome sequencing of tumor material [] to identify tumor mutations in a patient with metastatic breast cancer."); *id.* at .011 ("Between 3 and 20 sections were macrodissected depending on the ***tissue sample***'s size. DNA from FFPE sections was extracted with QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions.") (emphasis added).

7. Claim 8: The method of claim 1, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation.

213. Forshew in view of the skill and knowledge of a POSA renders obvious the method of claim 1, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation.

214. As described above with respect to claim 1, which requires targeted multiplex amplification to amplify 10 to 500 target loci, Forshew discloses performing TAm-Seq with larger numbers of amplicons and teaches that the degree of multiplex may be greater than ten, or even twenty. *See supra* Section IX.B.1.c.

215. Forshew discloses performing targeted multiplex amplification to amplify 20 and 48 target loci. Forshew discloses performing TAm-Seq with 48 sequence-specific primer pairs to amplify 5995 bases of genomic sequence covering coding regions (exons and exon junctions) of *TP53* and *PTEN*, as well as selected regions in *EGFR*, *BRAF*, *KRAS*, and *PIK3CA* (Table S1) by overlapping short amplicons (Fig. 1A). EX1003.003; *see also id.* at Table S1 (20 pairs of primers).

216. Forshew's Table S6 reports 23 point mutations (*i.e.*, SNVs), which were identified in plasma samples from ovarian cancer patients by performing TAm-Seq. *Id.* at .007 (Table 2) ("Mutations identified by TAm-Seq in a set of 62 plasma samples from ovarian cancer patients."), Table S6 ("Additional data for Table 2"). Table S6 includes "Gene" and "Location (build hg19) and base change" for various samples. *Id.* at Table S6. The "Location (build hg19) and base change" shows unique SNVs, demonstrating that there are 23 different point mutations (*i.e.*, SNVs) reported in Table S6. *Id.*

217. Accordingly, Forshew discloses and teaches performing TAm-Seq using higher numbers of amplicons, including within the claimed range of 20 to 50 target loci.

218. It would have been obvious to a POSA to use greater multiplex TAm-Seq because significantly greater multiplex amplification of DNA was already well-established in the art. This is evidenced by, for example, relevant prior art

pertaining to the preparation of DNA libraries, and in particular libraries to be used later for sequencing, including Patent Application US 2010/0120038 A1 (EX1083), which published May 13, 2010. EX1083 ¶ 119 (teaching a multiplex PCR amplification reaction using 9,216 primer pairs, to amplify 9,216 target sequences in the same reaction volume); *see also, e.g.*, Patent Application US 2012/0270212 A1 (EX1096) (published Oct. 25, 2012) ¶¶ 19, 64, 201, 223, 258 (performing multiplex PCR using targeted primer design of 10,000 loci); EX1040.007 ("To increase the throughput of PCR by keeping the number of PCR reactions as low as possible, there is the alternative of multiplex PCR (Figure 1.3b). Given careful primer design and reaction optimization, several dozen primer pairs can be used together effectively in a multiplexing reaction [32]. Indeed, software specifically created to help with multiplex PCR assay design is available [33].").

219. By Patent Owner's own admission, higher levels of multiplex were routine in the art. In the related '035 patent, Patent Owner describes that "the general belief in the art is that multiplexing PCR for sequencing is limited to about 100 assays in the same well." EX1049 at 48:25-29. Thus, a POSA would have been aware of how to perform multiplex PCR up to 100 assays in a single well, and would have been able to do so as a matter of routine.

220. Patent Owner's expert in the parallel district court litigation, Dr. Metzker, has also opined before the Board that years before 2015 it was known to

amplify up to 9,216 targets in a single tube for sequencing. *See* EX1082 ¶ 98 ("The pre-pre-amplification step uses PCR amplification with 9,216 primer pairs, to amplify 9,216 target sequences."). In the parallel district court litigation, Dr. Metzker reaffirmed his opinion:

> Q. Okay. So in the PCR -- in the Fluidigm reference that you opined upon, that is teaching a single-tube multiplex PCR of over 9,000 targets for sequencing; correct?
>
> A. It does have a preamplification step that does target at multiplex PCR.
>
> Q. Of over 9,000 targets; correct?
>
> A. That's what they report.

EX1052 at 87:24-88:6; *see also* EX1083 ¶ 119.

221. Thus, in my opinion, a POSA would have been motivated to apply routine techniques to enhance the multiplex capability taught in Forshew. A POSA would have done so to increase the number of gene loci targeted in TAm-Seq, allowing for the detection and tracking of a broader set of mutations. Further, given the known success of highly multiplexed PCR in preparation for sequencing, a POSA would have had a reasonable expectation of success.

8. Claim 9: The method of claim 1, wherein the targeted multiplex amplification amplifies 50 to 100 target loci each encompassing a different tumor-specific SNV mutation.

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222. Forshew in view of the skill and knowledge of a POSA renders obvious the method of claim 1, wherein the targeted multiplex amplification amplifies 50 to 100 target loci each encompassing a different tumor-specific SNV mutation.

223. As discussed above with respect to claims 1 and 8, which require targeted multiplex amplification of 10 to 500 and 20 to 50 target loci, respectively, Forshew teaches using TAm-Seq with 48 sequence-specific primer pairs to amplify 5995 bases of genomic sequence covering coding region (exons and exon junctions) of *TP53* and *PTEN*, and selected regions in *EGFR*, *BRAF*, *KRAS*, and *PIK3CA* (table S1) by overlapping short amplicons (Fig. 1A). EX1003.004.

224. For the same reasons as above, Forshew renders obvious amplifying higher numbers of target loci. *See supra* Section IX.B.7.

9. Claim 10: The method of claim 1, wherein the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample.

225. Forshew in view of the skill and knowledge of a POSA renders obvious the method of claim 1, wherein the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample.

226. As discussed above with respect to claim 1, which requires the generation of amplicons, Forshew teaches a targeted multiplex amplification

process, which required the design of PCR primers targeting the plurality of SNV mutations identified in the tumor sample. EX1003.016 ("[t]arget-specific primers were designed with universal primer sequences (termed CS1 and CS2) appended at the 5'-end (table S1)"; *id.* ("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."); *see also id.* at Figure S1 ("PCR strategy and primer design"). The targeted multiplex PCR reaction is also depicted and described in Figure 1B, wherein the "initial preamplification step was performed for 15 cycles using a pool of the ***target-specific primer pairs***." *Id.* at .003 (Fig. 1B legend) (emphasis added).

227. A POSA would have understood that targeted multiplex PCR amplification (including that disclosed by Forshew) inherently requires the design of PCR primers targeting the tumor-specific SNV mutations to be amplified. *See* EX1040.007 ("To increase the throughput of PCR by keeping the number of PCR reactions as low as possible, there is the alternative of multiplex PCR (Figure 1.3b). Given careful primer design and reaction optimization, several dozen primer pairs can be used together effectively in a multiplexing reaction [32]. Indeed, software specifically created to help with multiplex PCR assay design is available [33].")).

228. Therefore, claim 10 would have been obvious over Forshew in view of a POSA's knowledge.

10. Claim 11: The method of claim 1, wherein the method further comprises performing barcoding PCR prior to the sequencing.

229. Forshew discloses to a POSA the method of claim 1 further comprising performing barcoding PCR prior to the sequencing.

230. Forshew discloses performing PCR to attach barcodes prior to sequencing. Forshew discloses that "[p]latform-specific adaptors and barcodes are attached by PCR following the single-plex amplification step" (EX1003 at Table S2) and the sequencing adaptors are necessary for sequencing (*id.* at .004). Forshew also discloses performing barcoding PCR prior to sequencing because "[s]equencing adaptors were separately attached at either end and the products [then] mixed together, such that single-end sequencing generated separate sets of forward and reverse reads." *Id.* at .004. This is depicted in Figure S1, reproduced below:

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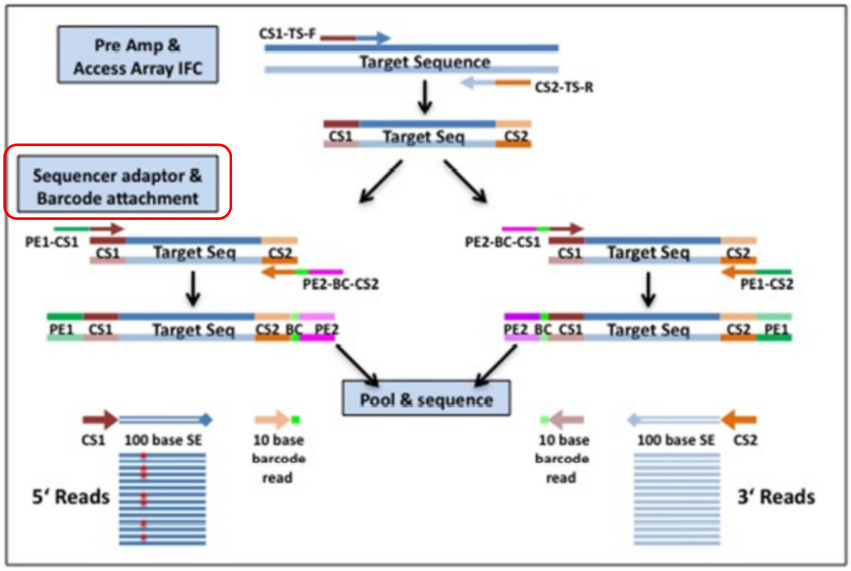

Figure S1: PCR strategy and primer design. Target-specific primers were synthesised with common adaptor sequences at their 5' ends (CS1 and CS2). These were used during the preamplification and single-plex stages to amplify the selected regions. Sequencing platform-specific adaptors and unique sample barcodes were attached during an additional round of PCR. The barcoding primers consist of Illumina sequencer adaptors (PE1 and PE2) at their 3' end, a unique 10 base barcode, and the common CS1 and CS2. Permutations of the universal tags and the sequencing adaptors enabled construction of bidirectional barcoded sequencing libraries. Using custom sequencing primers directed to the two tag sequences, sequence reads representing both strands of the amplicon pool were obtained from a single-end run. Both combinations were used in order to barcode and read amplicons in both directions. All samples were then pooled, cleaned, and then sequenced.

Id. at Fig. S1 ("The barcoding primers consist of Illumina sequencer adaptors (PE1 and PE2) at their 3' end, a unique 10 base barcode, and the common CS1 and CS2").

231. As discussed above in respect to claim 1, a POSA would also have found it obvious to include the sequence(s) that function as barcode(s) in the sequence-specific primers used in the targeted multiplex amplification to simplify the process of generating amplicons, as it would then only require a single PCR amplification reaction step. *See supra* Section IX.B.1.c. In doing so, the method would include performing barcoding PCR prior to sequencing.

232. Therefore, claim 11 would have been obvious over Forshew in view of a POSA's knowledge.

11. Claim 12: The method of claim 1, wherein the method further comprises detecting recurrence and/or metastases of the cancer from the SNV mutations detected in the cell-free DNA.

233. Forshew discloses to a POSA the method of claim 1, wherein the method further comprises detecting recurrence and/or metastases of the cancer from the SNV mutations detected in the cell-free DNA.

234. For example, Forshew teaches the "use of TAm-Seq to noninvasively identify the origin of metastatic relapse in a patient with multiple primary tumors." EX1003.002; *see also id.* ("We further used TAm-Seq to monitor tumor dynamics, and tracked 10 concomitant mutations in plasma of a metastatic breast cancer patient over 16 months"). Forshew also teaches that "a method that measures multiple mutations in parallel [when looking for recurrent or residual tumor] can have a low likelihood of a false-negative result even if the detection rate for each mutation is less than 50%." *Id.* at .011.

235. The ability to monitor mutations associated with cancers was well-known in the art. For example, Takano et al., *Epidermal Growth Factor Receptor Gene Mutations and Increased Copy Numbers Predict Gefitinib Sensitivity in Patients with Recurrent Non-Small-Cell Lung Cancer*, Journal of Clinical Oncology,

2005, Vol. 23, No. 28 (EX1104) ("Takano"), which published on October 1, 2005, expressly teaches that *EGFR* mutations in recurrent cancer can determine the clinical outcome for cancer patients treated with gefitinib. EX1104.001. Takano reported significantly better response rate, time to progression, and overall survival for patients with *EGFR* mutations compared to wild-type *EGFR*. *Id.* at .001, .005, .007 (Tables 5, 6). This shows that monitoring patients for the recurrence of *EGFR* mutations provides guidelines for cancer treatment. A POSA would have found it obvious to detect these mutations using the TAm-Seq method of Forshew and would have been motivated to do so because the presence of EGFR mutations was correlated with increased response to gefitinib.

236. Therefore, claim 12 would have been obvious over Forshew in view of a POSA's knowledge.

12. Claim 13: The method of claim 1, wherein the cancer is colorectal cancer, lung cancer, bladder cancer, or breast cancer.

237. Forshew discloses to a POSA the method of claim 1, wherein the cancer is colorectal cancer, lung cancer, bladder cancer, or breast cancer.

238. Forshew teaches performing whole genome sequencing and TAm-Seq to identify tumor mutations in a patient with breast cancer:

> 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. 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).

EX1003.010 (emphasis added).

239. Therefore, claim 13 would have been obvious over Forshew in view of a POSA's knowledge.

13. Claim 14:

a. [14.preamble]: A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample, the method comprising:

240. To the extent that the preamble of the claim is limiting, Forshew discloses a method of preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more SNV mutations in the plasma sample.

241. For the same reasons as described above for claim 1, these same recited elements are disclosed by Forshew. *See supra* Section IX.B.1.a.

242. Therefore, Forshew discloses the preamble of claim 14.

b. [14.a]: performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;

243. Forshew repeatedly discloses to a POSA performing whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations.

244. For the same reasons as described above for claim 1, these same recited elements are disclosed and taught to a POSA by Forshew. *See supra* Section IX.B.1.b.

c. [14.b]: performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons, wherein the target loci are amplified together in the same reaction volume; and

245. Forshew discloses to a POSA the method of using targeted multiplex amplification to amplify 10 to 500 target loci encompassing different tumor-specific SNV mutations from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons, wherein the target loci are amplified together in the same reaction volume.

246. Claim element 14.b is identical to claim element 1.b, except it does not require the amplicons to "hav[e] a length of 50-150 bases." For the same reasons

as above, these same recited elements are disclosed and taught to a POSA by Forshew. *See supra* Section IX.B.1.c.

d. [14.c]: sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads, wherein the method is capable of detecting an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus.

247. Forshew discloses the key components of this claim element to a POSA and teaches or suggests sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads, wherein the method is capable of detecting an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus.

248. Claim element 14.c is identical to claim element 1.c, except claim element 1.c recites "wherein the sequencing has a depth of read of at least 50,000 per target locus" whereas claim element 14.c recites "wherein the method is capable of detecting an SNV mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the SNV locus." For the reasons stated above in connection with claim element 1.c, the "sequencing" and "detecting" elements of this limitation are taught in Forshew. *See supra* Section IX.B.1.d.

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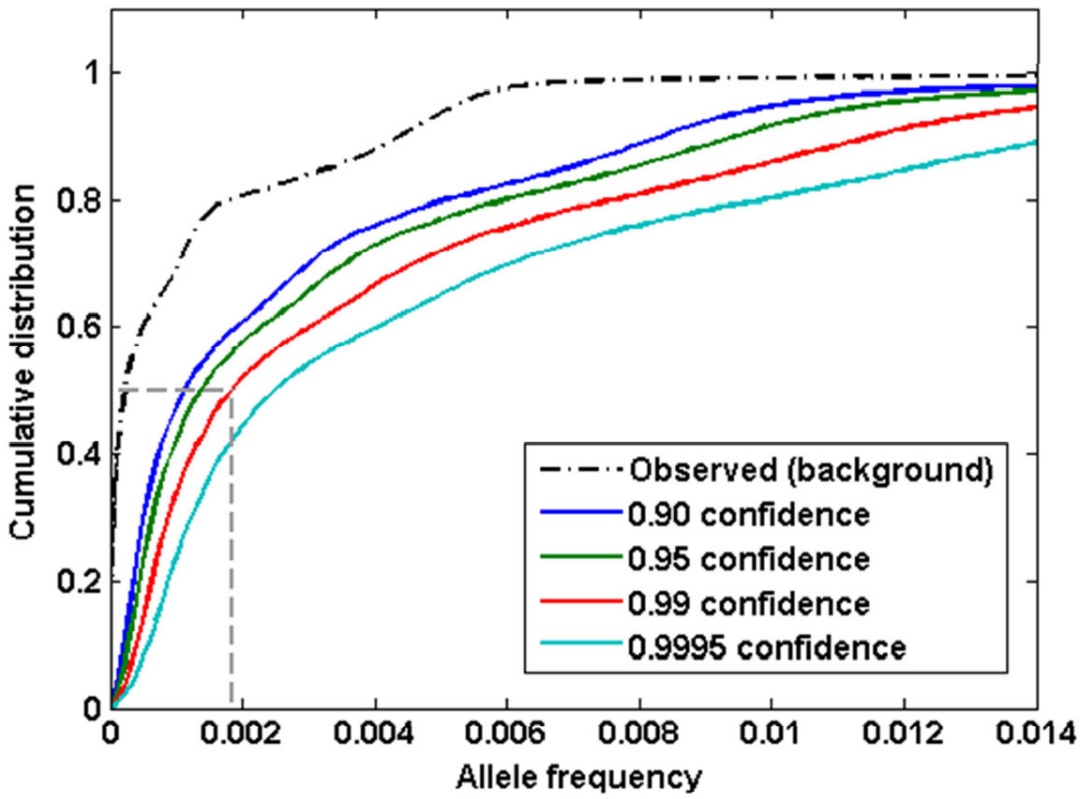
(i) A POSA Would Have Increased Sequencing Depth To Increase Sensitivity

249. A POSA would have been motivated 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, for the reasons set forth above with respect to claim 1. *See supra* Section IX.B.1.d. As described above, a POSA would have understood Forshew to disclose or teach increasing sequencing depth, reaching at least 50,000 per target locus, because it would improve detection of tumor-specific SNV mutations, allowing detection of cancer recurrence earlier in time and at lower levels, providing improved patient outcomes. *See supra id.* (citing EX1003.010). Claim 14 requires obtaining results like those that motivated the higher sequencing depth recited in claim 1, namely, increased sensitivity in detecting tumor-specific SNV mutations that allows earlier detection and better outcomes for patients.

250. 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, as discussed above. *See supra* Section IX.B.1.d; EX1003.011.

251. For example, Forshew describes detecting a median 0.03% background rate of non-reference (*i.e.*, mutant) reads. EX1003.004. Figure S3 shows frequencies that could be detected at lower rates with different confidence levels

above the background read rate, including allele frequencies of 0.015% and below (i.e., allele frequencies of 0.00015 and below):



Id. at Fig. S3 (showing frequency of detected non-reference alleles). In Figure S3, the mean background read rate for each one of the possible base substitutions is shown in black (cumulative distribution as function of allele frequency) and colored lines show the cumulative distribution of frequencies that would exceed the indicated confidence margins. *Id.* As explained in Forshew, "[w]hen…measur[ing] 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." *Id.* at .004. Forshew also discloses, as shown in Figure S3, 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)," that is, for example, that 50% of possible variants can be detected at a frequency of 0.14% or below with a confidence margin of 0.95. *Id.* at .004, Fig. S3. As can be read from the graph, a fraction of possible base substitutions can be detected at and below 0.015%, as depicted in the lower left-hand portion of the graph, illustrating lower, but non-zero, rates of detecting SNVs at lower confidence levels at such allele frequencies. *Id.*

252. As described above, Forshew further describes increasing sequencing depth or fidelity to improve detection of tumor-specific SNV mutations, allowing detection of cancer recurrence earlier in time and at lower levels. *Id.* at .011 ("higher read depth" allows "for enhanced mutation detection without change to protocols"). Further, Forshew identifies a number of other assays that can detect targeted loci down to 1 mutant allele in 10,000 or more wild-type alleles, corresponding to detecting an SNV mutation that is present in less than or equal to 0.01% of cfDNA. *Id.* at .010. Also, as Forshew teaches that this level of sensitivity has been met by a number of different methods, it is clear that a POSA

would have had a reasonable expectation of successfully achieving a sensitivity of 0.015% because this had already been accomplished. *Id.*

253. The well-established understanding of improving sensitivity by increasing sequencing depth and fidelity, demonstrated throughout the prior art, relied on by Patent Owner, and attested to by Patent Owner's experts, bears out that improving detection sensitivity was likewise well-known in the art. *See supra* Section IX.B.1.d. The '454 patent itself also lists 0.015% in lists of potential limits of detection without suggesting need for anything different or additional. EX1001 at 5:30-34, 6:20-21, 65:21-25, 92:58-93:10.

254. As discussed above, Forshew expressly teaches methods to increase the sensitivity of detection, including to increase both the sequencing depth and fidelity, but also including use of additional replicates (also amounting to increasing sequencing depth) and improved algorithms. EX.1003.011. Moreover, to the extent that Forshew itself is deemed to not obtain the sensitivity required, a POSA would have understood Forshew to not only to teach that the greater sensitivity was desired, but also to teach the use of methods to increase sensitivity, including those well-known in the art, that would have led to the desired level of sensitivity, particularly with improvements in the art up to the filing of the '703 application in 2015. Further, a POSA would have understood that achieving greater sensitivity by use of these methods was well-known in the art.

255. Obtaining greater sensitivity by increasing sequencing depth, for example, was well-known in prior art in addition to Forshew. *See supra* Section IX.B.1.d (citing EX1057 (teaching required depth of sequencing for detecting SNPs at levels recited in claim 14); EX1086 (disclosing detection of rare tumor mutations by using high sequencing depth); EX1062.010, Figure 2, legend ("the fraction of mutant DNA fragments dropped to 0.015%"); EX1090; EX1091.0036; EX1101; EX1105.001, Supplemental Fig. 2 (discussing correlation between increasing reads and increasing sensitivity); EX1108.011 (same).

256. Narayan, for example, teaches a detection limit of 0.014% for mutations in circulating tumor DNA through the use of DNA sequencing. *See* EX1086.004 ("Using this approach, we were able to reduce the frequency of miscalls of specific mutations from known wild-type samples to a median value of 0.014%"). As Narayan explains, the "deep sequencing approach described above shows the successful application of principles that we believe could form the basis of a practical diagnostic test to measure tumor-derived DNA levels in blood." *Id.* at .006. Patent Owner has likewise grounded its position in various cases and proceedings on higher sensitivity being desirable, and that it can be achieved by increasing sequencing depth. *See supra* Section IX.B.1.d.; EX1088.032-.033 ("[i]t was well-known by 2009 that the number of molecules analyzed is one component of sensitivity, and that ***higher sensitivity could be achieved by sequencing more***

molecules") (emphasis added), 27 ("And as discussed above, it was well-known by 2009 that ***by sequencing more molecules, sensitivity will necessarily increase***.") (emphasis added). Likewise, Patent Owner's experts in various proceedings have testified accordingly as to improving sensitivity by increasing sequencing depth. *See supra* Section IX.B.1.d.; EX1094 ¶ 119 (Dr. Quackenbush testifying in the case cited above that "POSAs prior to November 6, 2009 were well aware that sequencing more molecules, or increasing sequencing depth, improves sensitivity" and that "the detection limit is determined by sequencing error rate and sequencing read depth (i.e., how many molecules are sequenced), reflecting the fact that there was a ***well-known correlation between the number of molecules sequenced and sensitivity***.") (emphasis added); EX1084 at 98:10-99:6 (Dr. Quackenbush testifying on behalf of Natera that there was nothing "nonconventional" about increasing sequencing depth, that this is a "standard practice in many fields," and that "***to get higher sensitivity*** for detecting molecules, you simply ***sequence more molecules***.") (emphases added).

257. Also discussed above, Patent Owner's expert in parallel district court litigation, Dr. Metzker, has opined in a proceeding before the Board that a POSA would have understood, in 2010, how to increase accuracy of sequencing using the Illumina GA sequencer by increasing the sequencing depth. *See supra* Section IX.B.1.d.; EX1082 ¶¶ 91, 206, 213-214. As explained above, a POSA would have recognized increasing the accuracy of sequencing to also increase detection

sensitivity. *See supra* Section IX.B.1.d. As discussed above, in parallel district court litigation, Patent Owner has asserted that increasing sequencing depth in connection with the TAm-Seq process would have presented various challenges that would have dissuaded a POSA from pursuing improved detection sensitivity by this approach. *See supra* Section IX.B.1.d.

258. Dr. Metzker, for instance, opined on behalf of Patent Owner that "[p]rior to 2015 there were many challenges in using next generation sequencing." EX1081 ¶ 94. Dr. Metzker also opined that "[s]equencing each SNV locus 50,000 times or more…would not reasonably be expected to identify rare variants in low-abundance ctDNA above the background 'noise' from sequencing-related error rates." EX1054.012.

259. In my opinion, these positions as to the purported challenges in using NGS prior to 2015 and that sequencing each locus at a sequencing depth of 50,000 times or more purportedly would not reasonably be expected to identify rare variants in ctDNA due to background "noise" arising from sequencing-related error rates are without merit, as discussed above. *See supra* Section IX.B.1.d. Moreover, also as discussed above, it is directly contrary to Dr. Metzker's own testimony before the Board in an earlier case about how it would have been "obvious" to increase sequencing depth to enhance accuracy. EX1082 ¶ 214. Far from complaining about alleged "challenges" with NGS, Dr. Metzker previously testified on behalf of

Patent Owner that as of 2009 the literature taught that at "high sequence coverage ***all*** NGS platforms have ***excellent variant calling accuracy*** (>95%) as assessed by the detection of known SNP variants." *See id.* ¶ 213 (citing EX1042) (emphases added).

260. Likewise, as discussed above, in the North Carolina litigation, Dr. Metzker opined about the use of enzymes with built-in "proofreading" capabilities that would overcome PCR errors that would allegedly cause problems for sequencing. *See* EX1081 ¶ 304. Moreover, also discussed above, a POSA would have known to use proofreading polymerases with lower error rates, which were well-known, and would have had a reasonable expectation of success in improving the fidelity of amplification and significantly decreasing the error rate in generating amplicons, allowing greater sensitivity in detecting SNVs. *See supra* Section IX.B.1.d.i; EX1089; EX1080.004.

261. Sequencing fidelity would similarly not have posed a barrier to reaching the required sensitivity of detection in 2015. As discussed above, Forshew, in 2012, teaches that this level of sensitivity was available in the art. *See supra* Section IX.B.1.d.i.; EX1003.011. A POSA would have understood that Forshew would not have done so if there was a barrier that prevented increasing sensitivity of detection. Also, as discussed above, Dr. Metzker himself testifies that a POSA would have been aware of a reference that discusses sequencers with error

rates as low as 0.01%, which would be sufficient for detecting an SNV at a frequency of 0.01%. *See supra* Section IX.B.1.d.i.; EX1081 ¶ 94 (citing EX1067 at Table 3); EX1055 at 102:25-103:7, 101:25-102:23. Moreover, the relevant sequencing technology advanced between 2012 and 2015, becoming more accurate, more efficient, and less costly. *See supra* Section IX.B.1.d.i.; EX1078.002; EX1068.015; EX1119; EX1086.006.

262. In sum, there is no basis for any suggestion that sequencing technology in 2015 was so problematic that a POSA would have avoided increasing sequencing depth to improve accuracy.

263. Between 2012 and 2015, the relevant sequencing technology simply became more accurate, more efficient, and less costly, which would have only provided further motivation to increase sequencing depth.

(ii) A POSA Would Have Been Motivated to Modify Forshew To Omit Intermediate PCR Steps

264. For the reasons described above in connection with claim element 1.c, a POSA would have been motivated to modify Forshew so to directly sequence the amplicons and would have had a reasonable expectation of success. *See supra* Section IX.B.1.d.ii.

14. Claim 15: The method of claim 14, wherein the sequencing has a depth of read of at least 50,000 per target locus.

265. Forshew discloses to a POSA the method of claim 14, wherein the sequencing has a depth of read of at least 50,000 per target locus.

266. For the same reasons as described above for claim 1, claim 15 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.1.d.

15. Claim 16: The method of claim 14, wherein the cell-free DNA comprises circulating tumor DNA.

267. Forshew discloses to a POSA the method of claim 14, wherein the cell-free DNA comprises circulating tumor DNA.

268. For the same reasons as described above for claim 2, claim 16 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.2.

16. Claim 17: The method of claim 14, wherein the SNV mutations comprise one or more clonal SNV mutations.

269. Forshew discloses to a POSA the method of claim 14, wherein the SNV mutations comprise one or more clonal SNV mutations.

270. For the same reasons as described above for claim 3, claim 17 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.3.

17. Claim 18: The method of claim 14, wherein the SNV mutations comprise one or more subclonal SNV mutations.

271. Forshew discloses to a POSA the method of claim 14, wherein the SNV mutations comprise one or more subclonal SNV mutations.

272. For the same reasons as described above for claim 4, claim 18 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.4.

18. Claim 19: The method of claim 14, wherein the SNV mutations comprise one or more clonal SNV mutations and one or more subclonal SNV mutations.

273. Forshew discloses to a POSA the method of claim 14, wherein the SNV mutations comprise one or more clonal SNV mutations and one or more subclonal SNV mutations.

274. For the same reasons as described above for claim 5, claim 19 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.5.

19. Claim 20: The method of claim 14, wherein the tumor sample of the subject is a tumor tissue sample.

275. Forshew discloses to a POSA the method of claim 14, wherein the tumor sample of the subject is a tumor tissue sample.

276. For the same reasons as described above for claim 6, claim 20 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.6.

20. Claim 22: The method of claim 14, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation.

277. Forshew discloses to a POSA the method of claim 14, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation.

278. For the same reasons as described above for claim 8, claim 22 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.8.

21. Claim 23: The method of claim 14, wherein the targeted multiplex amplification amplifies 50 to 100 target loci each encompassing a different tumor-specific SNV mutation.

279. Forshew discloses to a POSA the method of claim 14, wherein the targeted multiplex amplification amplifies 50 to 100 target loci each encompassing a different tumor-specific SNV mutation.

280. For the same reasons as described above for claim 9, claim 23 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.9.

22. Claim 24: The method of claim 14, wherein the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample.

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281. Forshew discloses to a POSA the method of claim 14, wherein the method further comprises designing PCR primers or hybrid capture probes targeting the plurality of SNV mutations identified in the tumor sample.

282. For the same reasons as described above for claim 10, claim 24 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.10.

23. Claim 25: The method of claim 14, wherein the method further comprises performing barcoding PCR prior to the sequencing.

283. Forshew discloses to a POSA the method of claim 14, wherein the method further comprises performing barcoding PCR prior to the sequencing.

284. For the same reasons as described above for claim 11, claim 25 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.11.

24. Claim 26: The method of claim 14, wherein the method further comprises detecting recurrence and/or metastases of the cancer from the SNV mutations detected in the cell-free DNA.

285. Forshew discloses to a POSA the method of claim 14, wherein the method further comprises detecting recurrence and/or metastases of the cancer from the SNV mutations detected in the cell-free DNA.

286. For the same reasons as described above for claim 12, claim 26 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.12.

25. Claim 27: The method of claim 14, wherein the cancer is colorectal cancer, lung cancer, bladder cancer, or breast cancer.

287. Forshew discloses to a POSA the method of claim 14, wherein the cancer is colorectal cancer, lung cancer, bladder cancer, or breast cancer.

288. For the same reasons as described above for claim 13, claim 27 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.13.

26. Claim 28: The method of claim 14, wherein the method is capable of detecting an SNV mutation that is present in 0.005% to 0.015% of the cell-free DNA comprising the SNV locus.

289. Forshew discloses to a POSA the method of claim 14, wherein the method is capable of detecting an SNV mutation that is present in 0.005% to 0.015% of the cell-free DNA comprising the SNV locus.

290. Claim 28 is identical to the limitation in 14.c, "less than or equal to 0.015%," except that there is a lower bound of "0.005%." For the same reasons as described above with respect to claim element 14.c, which teaches detecting at least

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0.015%, claim 28 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.14.d.

291. Specifically, as discussed above, Narayan, using a high-fidelity polymerase (HiFi Hotstart, Kapa Biosystems), teaches a detection limit of 0.014% for mutations in ctDNA in plasma. *See supra* Section IX.B.14.d.

C. Claims 3-5, 7, 17-19 and 21 of the '454 Patent Are Obvious Over Forshew In View of Bai And/Or Bashashati, and A POSA's Knowledge

292. As described below, it is my opinion that claims 3-5, 7, 17-19, and 21 are unpatentable as obvious over Forshew in view of Bai and/or Bashashati and a POSA's knowledge.

293. A POSA seeking to practice Forshew's method in 2015 would have understood Forshew, Bai, and Bashashati to be in the same field of endeavor as the '454 patent. Each is directed to detecting and monitoring cancer mutations for improved detection, design of treatment, and prognosis. EX1001 at 24:63-65, 168:6-23; *see also id.* at 41:57-61, Figs. 51A-B; EX1003.002, .011; EX1005 at Abstract, .001-003, .006, Fig. 1; EX1004.001, Abstract, .007-.008. Moreover, the teachings of each of these references is reasonably pertinent to the particular problem that the '454 patent seeks to solve, detecting and monitoring mutations, i.e., SNVs, such that each of Forshew, Bai, and Bashashati would have commended itself to an inventor's attention in considering the problem addressed.

294. A POSA would have been motivated to combine the teachings of Forshew with those of Bai and/or Bashashati because determining the clonal heterogeneity of a tumor would have provided a more complete picture of the genetic makeup of the cancer, which would have aided in the task of monitoring and tracking the cancer and allowed for improved treatment. EX1003.002, .011; EX1005.006; EX1004.001, Abstract.

295. In sum, to a POSA, Forshew teaches or suggests determining heterogeneity of tumor samples which a POSA would have understood to be useful for improved monitoring and treatment, with a reasonable expectation of success, where both determining clonal heterogeneity and the benefits of doing so for improved monitoring and treatment were well-known in the art.

1. Claims 3 and 17: Clonal SNV Mutations

296. Claims 3 and 17 recite that "the SNV mutations comprise one or more clonal SNV mutations." As explained above, Forshew specifically discloses the detection of a clonal SNV mutation in the *TP53* gene. *See supra* Section IX.B.3. This clonal mutation was present in eight tissue regions that were sampled. *See id.*

297. To the extent Forshew is deemed to not disclose "clonal mutations," though, Forshew in view of Bai and/or Bashashati and the knowledge of a POSA discloses and teaches the method including one or more clonal SNV mutations.

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298. Specifically, as discussed above, a POSA would expect both clonal and subclonal mutations in a tumor. *See supra* Section IX.B.4 (citing Bai and Bashashati for clonal and subclonal mutations in tumors being well-known to a POSA).

299. Both clonal and subclonal mutations were well-known and common categories of cancer-related mutations. A POSA would have understood that to monitor and track cancer effectively would require identifying the various categories of cancer-related mutations, including clonal mutations. *See id.* This is clear from the prior art. For example, Bashashati teaches generally that "cancers are assumed to originate from a monoclonal composition (the ancestral clone), with subsequent evolution leading to selective expansions of genomically distinct subclones." EX1004.002.

300. Bashashati reports the distribution of different mutations (including clonal mutations) within the tumor at length. *See generally* EX1004; *id.* at .002 ("A panel of shared, identical mutations, indicative of a single ancestral clonal origin, was evident…"). Bai focuses on lung cancer, likewise explaining that the formation of lung cancer "is a multistage process by which monoclonal cancer cells gradually become heterogeneous owing to clonal evolution and genetic/epigenetic instability." EX1005.001.

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301. Thus, a POSA would have understood that cancer tumors generally will include both mutations that were present at the outset of tumor formation (clonal mutations), as well as later-arising variants (subclonal mutations) that develop over the lifecycle of the tumor. To effectively track and monitor cancer, a POSA would have been motivated to identify and detect the clonal mutations (as well as the subclonal mutations). A POSA would have had a reasonable expectation of successfully doing so because Forshew, Bashashati, and Bai all taught detection of clonal (and subclonal) cancer mutations generally in both tumor and cfDNA, including by performing sequencing. *See, e.g.*, EX1004.002 ("Next-generation sequencing technology has enabled the study and quantification of clonal evolution and intratumoural diversity in cancer….").

2. Claims 4 and 18: Subclonal SNV Mutations

302. Claims 4 and 18 recite that "the SNV mutations comprise one or more subclonal SNV mutations." As explained above, Forshew discloses the detection of a subclonal SNV mutation in the *EGFR* gene. *See supra* Section IX.B.4. This mutation in the *EGFR* gene was present in only two of the eight tissue regions that were sampled. *Id.*

303. To the extent Forshew is deemed to not disclose "subclonal mutations," though, Forshew in view of Bai and/or Bashashati and the knowledge of a POSA discloses and teaches the method including one or more subclonal SNV mutations.

304. As described above with respect to claims 3 and 17, Bashashati and Bai teach that tumors generally include both clonal and subclonal mutations. *See supra* Section X.C.1. As such, a POSA would have been motivated to track and detect subclonal mutations because they are an integral part of cancer tumors and knowledge about them would have informed diagnosis, monitoring, and treatment. Further, a POSA would have had a reasonable expectation of successfully tracking and detecting subclonal mutations. Bashashati identified and tracked subclonal mutations. *See, e.g.*, EX1004.002 ("In addition, for a subset of cases (four patients), plasma (pre-operative and pre-anaesthetic) was used to identify the presence of clonally dominant and subclonal mutations through deep sequencing of ctDNA."); *id.* at .005 ("Mutational events in the ancestral clone are detectable in cell-free circulating tumour DNA from plasma."); *id.* at .006 ("Observations of subclonal NF1 deletions reinforced evidence from the mutation data that important driver mutations may be acquired late in the evolutionary history of the tumour.").

3. Claims 5 and 19: Clonal and Subcloncal SNV Mutations

305. Claims 5 and 19 recite that "the SNV mutations comprise one or more clonal SNV mutations and one or more subclonal SNV mutations." As explained above, Forshew specifically discloses the detection of a clonal SNV mutation in the *TP53* gene. *See supra* Section IX.B.3. This clonal mutation was present in eight tissue regions sampled. *See id.* As explained above, Forshew discloses detection

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of a subclonal SNV mutation in the *EGFR* gene. *See supra* Section IX.B.4. This mutation was present in only two of the eight tissue regions that Forshew sampled. *See id.*

306. To the extent Forshew is deemed to not disclose one or more clonal SNV mutations and one or more subclonal mutations, though, Forshew in view of Bai and/or Bashashati and the knowledge of a POSA discloses and teaches the method including one or more clonal and subclonal SNV mutations.

307. As described above with respect to claims 3 and 17 (*see supra* Section IX.C.1) and claims 4 and 18 (*see supra* Section IX.C.2), Bashashati and Bai teach that tumors generally include both clonal and subclonal mutations. As also described above, a POSA would have been motivated to track and detect clonal and subclonal mutations because they are an integral part of cancer tumors and would have had a reasonable expectation of successfully doing so. *See supra* Sections IX.C.1-2.

4. Claims 7 and 21: Clonal Heterogeneity

308. Claims 7 and 21 recite determining "clonal heterogeneity of the tumor sample," which relates to determining the extent to which different regions of a tumor are composed of different variations of that particular cancer.

309. As the '454 patent explains, "many cancers are heterogeneous meaning that different cells in a tumor are genetically distinct from other cells in the same

tumor." EX1001 at 24:63-65. The '454 patent also discloses an example of assessing "tumor heterogeneity" in its Example 13 where samples from various "regions" of a cancerous lung are collected and then analyzed by "whole exome sequencing" to determine the relative amounts of mutations present in each region. *Id.* at 168:6-23; *see also id.* at 41:57-61, Figs. 51A-B. Specifically, the '454 patent discloses "isolat[ing] ctDNA and identify[ing] both clonal and subclonal SNV mutations in plasma to overcome tumor heterogeneity" where the variant allele frequency (VAF) was determined for different genes and samples, including the tumor heterogeneity in primary tumors. EX1001 at 42:59-62, 41:65-42:6, Figs. 51B, 53A-B, 54A. Figure 54A, for example, "is a plot of % VAF in Primary Lung Tumor." *Id.* at 42:4, Fig. 54A.

310. It was well-known before 2015 that tumor tissue is genetically heterogeneous. *See, e.g.*, EX1003.002, .011; EX1005.002-003, Figs. 1 and 2; EX1004.001, .007-.008.

311. Forshew teaches that "mutation profiles in different metastatic clones can differ significantly from each other or the parent primary tumor" and that "[e]volutionary changes within the cancer can alter the mutational spectrum of the disease and its responsiveness to therapies," such that there was a need to identify the range of mutations present in a cancer patient to improve monitoring and treatment. EX1003.002, .011; *see also supra* Sections IX.B.3-5.

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312. Forshew teaches determining the allele frequency (AF) in the ovarian cancer formalin-fixed, paraffin-embedded (FFPE) tissue samples:

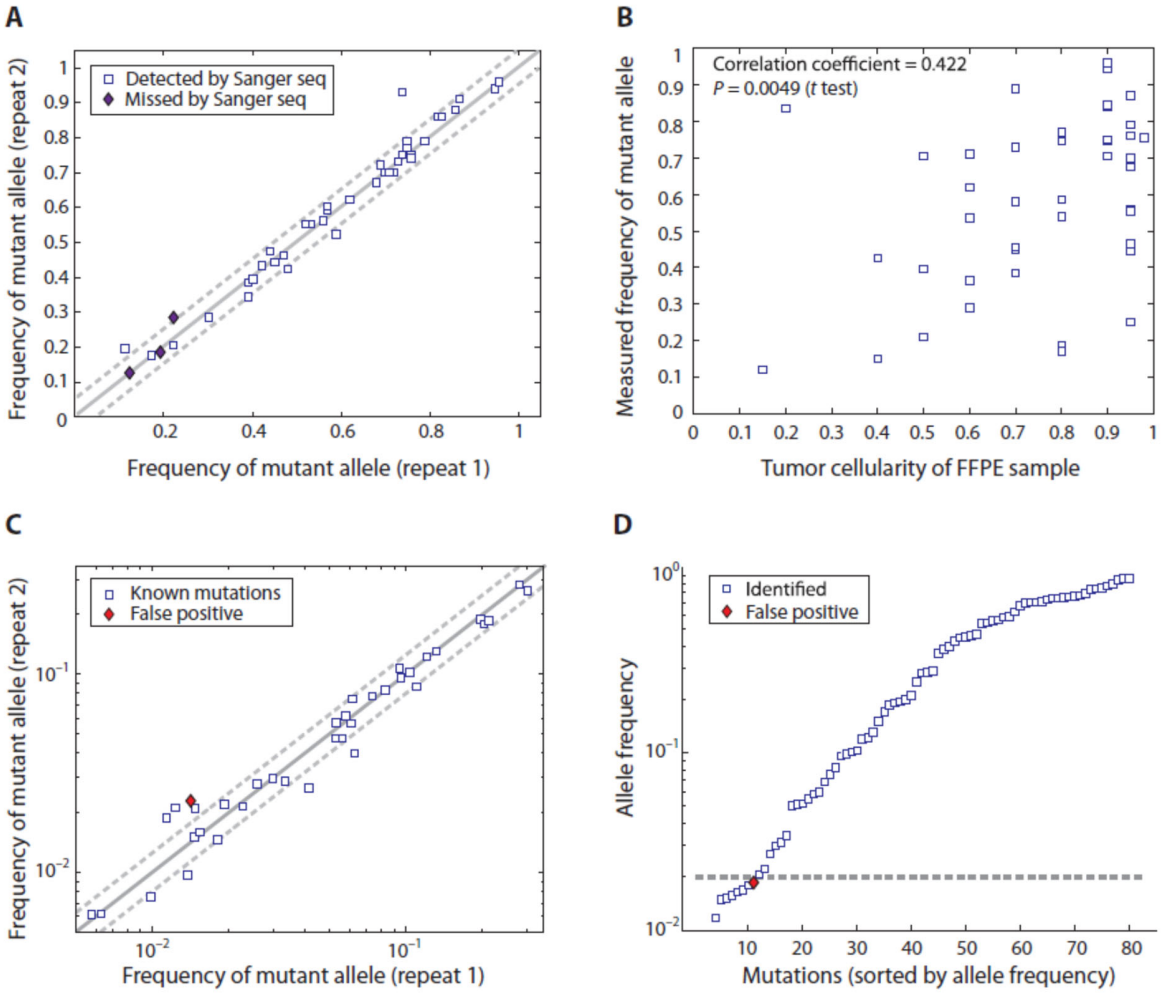


Fig. 2. Identification of mutations in ovarian cancer FFPE samples by TAm-Seq. (**A**) Concordance between duplicate measurements of AFs of mutations identified in fragmented DNA extracted from FFPE samples. The mutation frequency in each library was calculated as the fraction of reads with the mutant (nonreference) base. Solid line indicates equality. Dotted lines indicate a difference in AF of 0.05. (**B**) Correlation of AF with FFPE tumor cellularity. The measured mutant AF (average of both repeats) correlated significantly with the cellularity, estimated from histology (table S3). (**C**) Concordance between duplicate measurements of AFs of mutations identified in a mixture of DNA extracted from different FFPE samples. (**D**) Summary of mutations called in FFPE using TAm-Seq, sorted by increasing AF. Dotted line indicates AF of 2%.

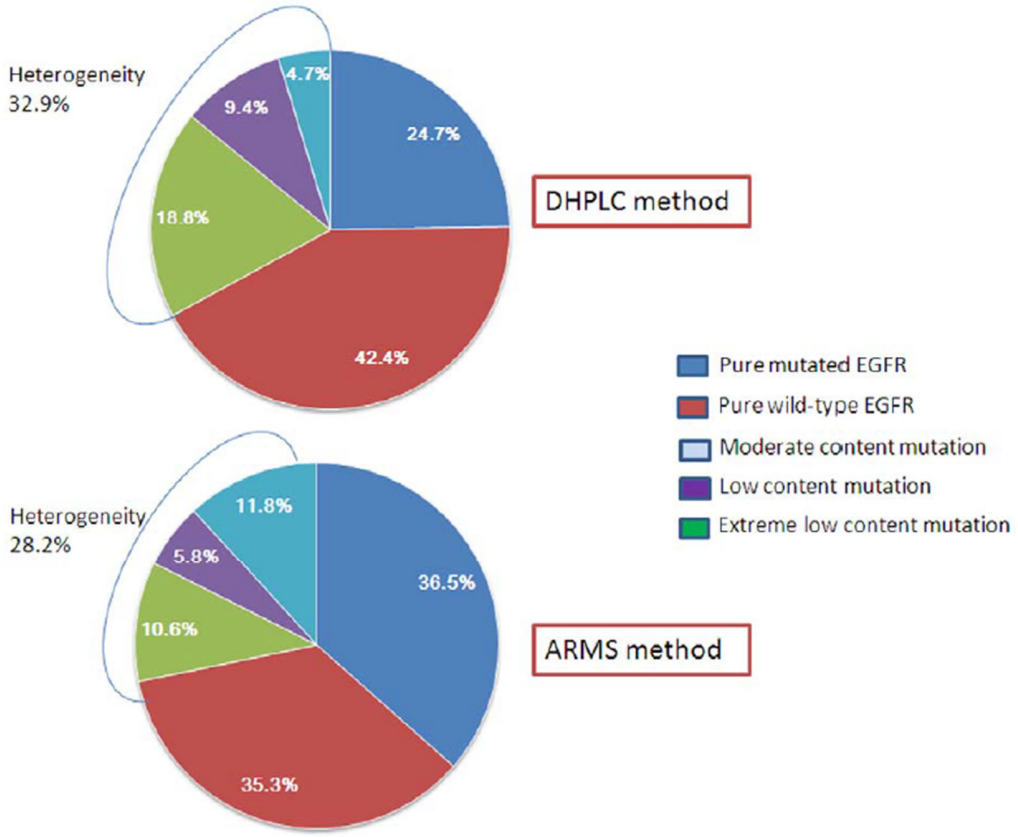
EX1003 at Fig. 2 (showing allele frequencies determined in ovarian cancer tissue samples).

313. Forshew also teaches determining the heterogeneity of tumor samples, from an ovarian cancer patient, by determining allelic frequency of the *TP53* mutation in samples from left and right ovaries and omental mass. *Id.* at Fig. 4A, legend (reporting retrospective analysis, including of "biopsy specimens collected at initial surgery…from patient 27 (Tables 1 and 2)"), Table 1 (noting that "[b]oth a *TP53* and an EGFR mutation were identified in this sample" from patient 27). Figure 4(A) reports 85% allelic frequency in the left and right ovaries and 43% allelic frequency in the omental mass. *Id.* at Fig. 4A, Fig. 4A, legend ("Percentages indicate mutant AFs."). A POSA would have understood this as establishing the heterogeneity of alleles present within tumor tissue. A POSA would have further understood this to teach the heterogeneity of alleles present in different portions of the same tumor, as tumors were understood to arise from single ancestral cells that acquire mutations. *See supra* Section VI.F. (citing EX1039.001).

314. Methods of determining clonal heterogeneity of tumor samples were also well-known in the art, and a POSA would have also found it obvious to combine Forshew with prior art disclosures of those methods.

315. Bai teaches that tumor samples obtained from the same individual show mutational heterogeneity with respect to *EGFR* mutations in different portions of the same tumor. EX1005.002-.003, Figs. 1 and 2. Bai discloses that "[m]utant frequencies varied widely throughout individual tumors, ranging between 5%-100%

by DHPLC and 1%-100% by ARMS." *Id.* at .003. Bai further reports that a percentage of "samples were identified to carry intratumoral *EGFR* mutational heterogeneity by DHPLIC and ARMS." *Id.* at .003. The percentage of heterogeneity of *EGFR* mutations is shown in Figure 1, reproduced below:



Id. at Fig. 1.

316. Bai also discloses benefits of determining clonal heterogeneity teaching that "mutational heterogeneity may be able to explain resistance mechanism of EGFR-TKI" and that there may be advantages to biopsies relative to single point or single time biopsies. *Id.* at .006.

317. Bashashati teaches determining clonal heterogeneity of tumors in its disclosure of characterizing and monitoring tumor cells in cancer patients. For example, Bashashati identified clonal heterogeneities in tumor samples and relied on these in determining the origin and nature of various tumors. EX1004.008 ("Results from PIK3CA mutation sequencing suggested the [fallopian tube] lesion in case 4 was composed of heterogeneous subclones" with further analysis revealing "two coexisting populations of nuclei in the [fallopian tube] lesion."); *see also id.* at .007-.008 (determining a fallopian tube lesion harbored mutations not present in all samples and determining phylogenetic relationships, and determining likely origin on particular tumor as metastatic implant). These different mutations were tracked in cfDNA using personalized NGS assays, thus monitoring clonal heterogeneity. *Id.* at .005 ("Cases 1 and 5 were enriched for the presence of mutations in the plasma that were also present in all intrapatient tumour samples, and thus part of the ancestral clone (Fisher exact test, $p < 0.05$; Figure 2).").

318. Bashashati also shows that a POSA would have appreciated the benefit of monitoring clonal heterogeneity of the tumor sample for improved treatment. *See, e.g.*, *id.* at .001, Abstract ("[W]e observed mixed cell populations in the early fallopian tube lesion….Our results revealed that [high-grade serous ovarian cancers] exhibit highly individual evolutionary trajectories and diverse genomic tapestries prior to therapy, exposing an essential biological characteristic to inform future

design of personalized therapeutic solutions and investigation of drug-resistance mechanisms.").

D. Claims 14-20, 22-28 of the '454 Patent Are Obvious Over Forshew In View of Bentley and/or Narayan, and A POSA's Knowledge

1. Claim 14

319. As described above in Section IX.B.13, claim 14 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Section IX.B.13. Additionally, for the reasons described above as well as the reasons described in this section, claim 14 would have been obvious over Forshew in view of Bentley and/or Narayan, and a POSA's knowledge. *Id.*

320. Narayan teaches a "deep sequencing method that demands redundancy within each clonal sequence to produce extremely high quality base calls in short, mutation-prone regions of plasma DNA." EX1086.001. This method was designed to overcome limitations of next generation sequencing in calling mutant variants found circulating in plasma cfDNA. *Id.* The depth of sequencing disclosed extends to "a median depth of 108,467 reads per locus" and the base call quality "reduc[es] the frequency of miscalls…to a median value of 0.014%." *Id.* at .003-.004.

321. Bentley discloses a relationship between the "Number of Reads" and the "Minimum frequency of SNP in population detectable with 95%…[and] 99%

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confidence." EX1057 ¶ 88. Specifically, sequencing a greater number of reads allows for detecting lower frequency SNVs. *Id.* ¶ 85. Bentley shows the results of these routine adjustments in Table 2:

TABLE 2

SNP Classes	Number of Reads	Minimum frequency of SNP in population detectable with 95% confidence	Minimum frequency of SNP in population detectable with 99% confidence
1	200000	0.002%	0.003%
2	100000	0.005%	0.007%
5	40000	0.014%	0.018%
10	20000	0.028%	0.037%
50	4000	0.14%	0.18%
100	2000	0.28%	0.37%
200	1000	0.55%	0.74%
500	400	1.39%	1.85%
1000	200	2.76%	3.64%

EX1057 ¶ 88 (Table 2). Bentley shows that sequencing to a depth of 40,000 would detect SNPs between a frequency of 0.014% and 0.018%, with 95-99% confidence. *Id.*

322. A POSA seeking to practice Forshew's method in 2015 would have understood Forshew, Bentley, and Narayan to be in the same field of endeavor as the '454 patent. Each is directed to detecting rare mutations using sequencing following DNA amplification. EX1001 at 24:63-65, 168:6-23; *see also id.* at claim 14 (describing method for detection of DNA variants "present in less than or equal

to 0.015% of the cell-free DNA" following amplification and sequencing); EX1003.002-.003 ("We applied this technique for detection of…rare mutations in circulating DNA from blood plasma."), .004 ("To characterize the quantitative accuracy of TAm-Seq as applied to circulating DNA, we simulated rare circulating tumor mutations by mixing plasma DNA from two healthy individuals."), 9 ("Detection of rare mutations in circulating DNA has long been pursued owing to its potentially transformative impact on cancer diagnosis and management."), 10 (discussing how to enhance mutation detection); EX1057 ¶ 90 ("This HLA sequencing system can also detect chimeric mixtures, e.g., the detection of the rare non-transmitted maternal allele present in the blood of SCID patients as referenced above."), *id.* ("In the described invention, the system is sufficiently high throughput to enable a complete, 8-locus HLA typing for multiple individuals, e.g., 24, 48, or more subjects, in a single sequencing run using a next-generation sequencing platform as described herein."); *id.* at Abstract (describing a "method for detecting one or more HLA sequence types," including "amplifying a plurality of first amplicons from a double stranded nucleic acid sample"); EX1086.003-.004 (describing method, including amplification and sequencing, "permitting detection and quantitation of rare mutations").

323. Moreover, both Forshew and Narayan are also directed to detecting and monitoring cancer-related SNV mutations, and the teachings of Bentley are

reasonably pertinent to the problem of detecting and monitoring SNVs to improve detection threshold and treatment recommendation, such that it also would have commended itself to an inventor's attention in considering the problem of monitoring cancer-related mutations.

324. Further, the '454 patent, Forshew, and Narayan all specifically concern cfDNA, and the '454 patent, Forshew, and Bentley all concern the detection of SNVs/SNPs. EX1001 at claim 14; EX1003.004; EX1057 ¶ 88 ("Table 2 displays the number of SNPs that can be screened…with the minimum allelic frequencies detectable at 95% and 99% confidence."); EX1086.003. Moreover, the teachings of each of these references are reasonably pertinent to the particular problem that the '454 patent seeks to solve, such that each of Forshew, Bentley, and Narayan would have commended itself to an inventor's attention in considering the problem addressed.

325. A POSA would have been motivated to combine the teachings of Forshew with those of Bentley and/or Narayan to attain the level of sensitivity to detect an SNV mutation present in less than or equal to 0.015% of the cfDNA comprising the SNV locus, because Bentley and Narayan would have provided useful information on the sensitivity that could be achieved with the TAm-Seq protocol disclosed by Forshew. EX1003.011 ("Higher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation

detection without change to protocols."). In particular, Narayan, teaches that a detection limit of 0.014% for mutations in circulating tumor DNA can be reached through the use of DNA sequencing methods like TAm-Seq. *See* EX1086.004 ("Using this approach, we were able to reduce the frequency of miscalls of specific mutations from known wild-type samples to a median value of 0.014%").

326. Further, Bentley teaches that a sequencing depth of 40,000 would, with 95-99% confidence, successfully detect SNPs between a frequency of 0.014% and 0.018%. EX1057 ¶ 88. Bentley also teaches that a sequencing depth of 100,000 would, with 95-99% confidence, successfully detect SNPs between a frequency of 0.005 and 0.007%. *Id*.

327. In sum, to a POSA, Bentley and Narayan would have provided a reasonable expectation of success that the TAm-Seq method of Forshew could detect SNV mutations that are present in less than or equal to 0.015% of cfDNA. *See* EX1003.011 ("Higher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols.").

328. For these as well as the reasons described above in Section IX.B.13, claim 14 would have been obvious over Forshew in view of Bentley and/or Narayan and a POSA's knowledge. *See supra* Section IX.B.13.

2. Claims 15-20 and 22-28

329. For the same reasons as described above in Section IX.D.1 for claim 14, as well as the reasons described above in Sections IX.B.14-IX.B.19 and IX.B.20-IX.B.26, claims 15-20 and 22-28 would have been obvious over Forshew in view of Bentley and/or Narayan, and a POSA's knowledge.

E. Claims 17-19 And 21 Are Obvious Over Forshew In View Of Bai and/or Bashashati and Bentley and/or Narayan, and A POSA's Knowledge

330. As described above in Sections IX.B.16-18, claims 17-19 would have been obvious over Forshew in view of a POSA's knowledge. *See supra* Sections IX.B.16-18. As described above in Section IX.C, claims 17-19 and 21 would have been obvious over Forshew in view of Bai and/or Bashashati, and a POSA's knowledge. *See supra* Section IX.C. As described above in Section IX.D.2, claims 17-19 would have been obvious over Forshew in view of Bentley and/or Narayan, and a POSA's knowledge. *See supra* Section IX.D.2. Additionally, for the reasons described above as well as the reasons described in this section, claims 17-19 and 21 would have been obvious over Forshew in view of Bai and/or Bashashati and Bentley and/or Narayan, and a POSA's knowledge. *See supra* Sections IX.B.16-18, IX.C., IX.D.2.

331. As described above, Narayan teaches a "deep sequencing method that demands redundancy within each clonal sequence to produce extremely high quality

base calls in short, mutation-prone regions of plasma DNA." EX1086.001. This method was designed to overcome limitations of next generation sequencing in calling mutant variants found circulating in plasma cfDNA. *Id*. The depth of sequencing disclosed extends to "a median depth of 108,467 reads per locus" and the base call quality to "reduc[ing] the frequency of miscalls…to a median value of 0.014%." *Id.* at .003-.004.

332. Bentley discloses a relationship between the "Number of Reads" and the "Minimum frequency of SNP in population detectable with 95%…[and] 99% confidence." EX1057 ¶ 88. Specifically, sequencing a greater number of reads allows for detecting lower frequency SNVs. *Id.* ¶ 85. Bentley shows the results of these routine adjustments in Table 2:

TABLE 2

SNP Classes	Number of Reads	Minimum frequency of SNP in population detectable with 95% confidence	Minimum frequency of SNP in population detectable with 99% confidence
1	200000	0.002%	0.003%
2	100000	0.005%	0.007%
5	40000	0.014%	0.018%
10	20000	0.028%	0.037%
50	4000	0.14%	0.18%
100	2000	0.28%	0.37%
200	1000	0.55%	0.74%
500	400	1.39%	1.85%
1000	200	2.76%	3.64%

EX1057 ¶ 88 (Table 2). Bentley shows that sequencing to a depth of 40,000 would detect SNPs between a frequency of 0.014% and 0.018%, with 95-99% confidence. *Id*.

333. A POSA seeking to practice Forshew's method in 2015, including as modified by the teachings of Bai and/or Bashashati to monitor clonal SNVs, subclonal SNVs, both clonal and subclonal SNVs, and clonal heterogeneity of the tumor sample, would have understood Forshew, Bai, Bashashati, Bentley, and Narayan to be in the same field of endeavor as the '454 patent and/or reasonably pertinent to the problem the inventor was seeking to solve such that it would have commended itself to the inventor's attention in considering the problem addressed. As discussed above, the '454 patent and each of Bai, Bashashati, and Narayan are in the same field of endeavor, detecting and monitoring cancer mutations for improved detection, design of treatment, and prognosis (*see supra* Sections IX.C., IX.D.1), and Bentley is reasonably pertinent to detecting cancer mutations at low levels, such that it would have commended itself to an inventor's attention in addressing the problem (*see supra* Section IX.D.1). Also as discussed above, the '454 patent and each of Bashashati, Bentley, and Narayan are in the same field of endeavor, detecting rare mutations using sequencing following DNA amplification, and Bai is reasonably pertinent to this problem in its teachings relating to clonal and subclonal evolution and mutational heterogeneity. *See supra* Sections IX.C., IX.D.1.

334. A POSA would have been motivated to combine the teachings of Forshew as modified by the teachings of Bai and/or Bashashati with those of Bentley and/or Narayan to detect SNV mutations (including clonal SNVs, subclonal SNVs, and clonal heterogeneity of the tumor sample) and to attain the level of sensitivity to detect a SNV mutation present in less than or equal to 0.015% of the cfDNA comprising the SNV locus.

335. As explained above, Forshew specifically discloses the detection of a clonal SNV mutation in the *TP53* gene, present in eight tissue regions sampled. *See supra* Section IX.B.3. As also explained above, Forshew discloses detection of a subclonal SNV mutation in the *EGFR* gene, present in only two of the eight tissue regions that Forshew sampled. *See supra* Section IX.B.4.

336. To the extent Forshew is deemed to not disclose one or more clonal SNV mutations and one or more subclonal mutations, though, Forshew in view of Bai and/or Bashashati and the knowledge of a POSA discloses and teaches the method including one or more clonal and subclonal SNV mutations. As described above (*see supra* Sections IX.C.1-2), Bashashati and Bai teach that tumors generally include both clonal and subclonal mutations. As also described above, a POSA would have been motivated to track and detect clonal and subclonal mutations because they are an integral part of cancer tumors and would have had a reasonable expectation of successfully doing so. *See supra* Sections IX.C.1-2. Similarly, as

described above, a POSA would have been motivated to combine the teachings of Forshew with those of Bai and/or Bashashati because determining the clonal heterogeneity of a tumor would have provided a more complete picture of the genetic makeup of the cancer, which would have aided in the task of monitoring and tracking the cancer and allowed for improved treatment. *See supra* Section IX.C.4.

337. Forshew discloses that "[h]igher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols." EX1003.011. Bentley and Narayan would have provided useful information on the sensitivity that could be achieved with the TAm-Seq protocol disclosed by Forshew. In particular, Narayan teaches that a detection limit of 0.014% for mutations in circulating tumor DNA can be reached through the use of DNA sequencing methods like TAm-Seq. *See* EX1086.004 ("Using this approach, we were able to reduce the frequency of miscalls of specific mutations from known wild-type samples to a median value of 0.014%"). Further, Bentley teaches that a sequencing depth of 40,000 would, with 95-99% confidence, successfully detect SNPs between a frequency of 0.014% and 0.018%. EX1057 ¶ 88. Bentley also teaches that a sequencing depth of 100,000 would, with 95-99% confidence, successfully detect SNPs between a frequency of 0.005 and 0.007%. *Id*.

338. In sum, to a POSA, Bentley and Narayan would have provided a reasonable expectation of success that the TAm-Seq method of Forshew could detect SNV mutations that are present in less than or equal to 0.015% of cfDNA. *See* EX1003.011 ("Higher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols.").

339. For these as well as the reasons described above in Sections IX.B.16-18, IX.C.1-4, and IX.D.2, claims 17-19 and 21 would have been obvious over Forshew in view of Bai and/or Bashashati, Bentley and/or Narayan, and a POSA's knowledge. *See supra* Sections IX.B.16-18, IX.C.1-4, IX.D.2.

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DECLARATION IN LIEU OF OATH

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1034 of Title 18 of the United States Code.

Dated: December 19th, 2023

By:__ _____

Niall Lennon