UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

FOUNDATION MEDICINE, INC.,
Petitioner,

v.

GUARDANT HEALTH, INC.,
Patent Owner.

Case IPR2019-00634
U.S. Patent No. 9,840,743

PETITIONER'S OPPOSITION TO PATENT OWNER'S MOTION TO AMEND

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Regulations

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EX1002.600

EXHIBIT LIST

Exhibit No.	Description
1001	U.S. Patent No. 9,840,743 ("the '743 patent")
1002	Declaration of Dr. Stacey Gabriel ("Gabriel Declaration")
1003	*Curriculum Vitae* of Dr. Stacey Gabriel
1004	U.S. Patent Application No. 15/467,570, March 23, 2017, Certification and Request for Prioritized Examination Under 37 C.F.R. § 1.102(e)
1005	U.S. Patent Application No. 15/467,570, April 27, 2017 Decision Granting Request for Prioritized Examination
1006	U.S. Patent Application No. 15/467,570, Original Claims as filed
1007	U.S. Patent Application No. 15/467,570, April 20, 2017, Preliminary Amendment
1008	U.S. Patent Application No. 15/467,570, May 10, 2017, Preliminary Amendment
1009	U.S. Patent Application No. 15/467,570, June 1, 2017, First Action Interview Pilot Program Pre-Interview Communication/Office Action
1010	U.S. Patent Application No. 15/467,570, June 29, 2017, Response to Pre-Interview First Office Action
1011	U.S. Patent Application No. 15/467,570, July 13, 2017, Applicant-Initiated Interview Summary
1012	U.S. Patent Application No. 15/467,570, August 4, 2017, Notice of Allowance

1013	U.S. Patent Application No. 15/467,570, August 29, 2017, Corrected Notice of Allowability
1014	U.S. Patent Application No. 15/467,570, September 5, 2017, Amendment After Notice of Allowance
1015	U.S. Patent Application No. 15/467,570, September 12, 2017, Corrected Notice of Allowability
1016	U.S. Patent Application No. 15/467,570, September 12, 2017, Amendment After Notice of Allowance
1017	U.S. Patent Application No. 15/467,570, September 1, 2017, Applicant-Initiated Interview Summary
1018	Nawroz et al. (1996) "Microsatellite alterations in serum DNA of head and neck cancer patients", Nature Medicine, 2(9), 1035-1037 ("Nawroz")
1019	U.S. Patent Application No. 15/467,570, September 21, 2017, Response to September 12 Amendment
1020	U.S. Patent Application No. 15/467,570, October 20, 2017, Amendment
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1022	Chiu et al. (2008), "Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma," Proc. Natl. Acad. Sci. USA, 2008 105(51), 20458-20463 ("Chiu")
1023	Kinde et al. (2011) "Detection and quantification of rare mutations with massively parallel sequencing," Proc. Natl. Acad. Sci. USA, 108(23), 9530-9535 ("Kinde 2011")

1024	Bianchi et al. (2012) "Genome-Wide Fetal Aneuploidy Detection by Maternal Plasma DNA Sequencing," Obstetrics & Gynecology, 119(5), 1-12 ("Bianchi")
1025	Chiu et al. (2011) "Non-invasive assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study," BMJ, 342:c7401 ("Chiu 2011")
1026	Sehnert et al. (2011) "Optimal Detection of Fetal Chromosomal Abnormalities by Massively Parallel DNA Sequencing of Cell-free DNA from Maternal Blood," Clinical Chemistry, 57:7, 1042-1049
1027	"Control," Oxford Dictionary of Biochemistry and Molecular Biology (2d ed. 2006), 143
1028	Redon et al. (2006) "Global variation in copy number in the human genome," Nature, 444 444-454 ("Redon")
1029	Gordon et al.(2012) "Causes and consequences of aneuploidy in cancer," Nature Reviews,13, 189-203 ("Gordon")
1030	Nielsen et al. (2011) "Genotype and SNP calling from next-generation sequencing data," Nat. Rev. Genet., 12(6), 443-451 ("Nielsen")
1031	Mertes et al. (2011) "Targeted enrichment of genomic DNA regions for next-generation sequencing," Briefings in Functional Genomics, 10(6), 374-386 ("Mertes")
1032	Fu et al., (2011) "Counting individual DNA molecules by the stochastic attachment of diverse labels," Proc. Natl. Acad. Sci. USA, 108(22), 9026-9031 ("Fu")
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1035	Forshew et al. (2012) "Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA," Sci.. Transl. Med., 2012, 4(136), 1-12 ("Forshew")
1036	U.S. Patent Application No. 15/467,570, Application Data Sheet
1037	Rizzo et al. (2012) "Key Principles and Clinical Applications of '*Next Generation*' DNA Sequencing," Cancer Prev. Res., 5, 887-900 ("Rizzo")
1038	Li et al. (2008) "Mapping short DNA sequencing reads and calling variants using mapping quality scores," Genome Research, 18, 1851-1858 ("Li")
1039	Lo et al. (1998) "Quantitative Analysis of Fetal DNA in Maternal Plasma and Serum: Implications for Noninvasive Prenatal Diagnosis," Am. J. Hum. Genet., 62, 768-775 ("Lo 1998")
1040	Narayan et al. (2012) "Ultrasensitive Measurement of Hotspot Mutations in Tumor DNA in Blood Using Error-Suppressed Multiplexed Deep Sequencing," Can. Res., 72(14), 3492-3498 ("Narayan")
1041	Kinde et. al. (2012) "FAST-SeqS: A Simple and Efficient Method for the Detection of Aneuploidy by Massively Parallel Sequencing," PLoS ONE, 7(7), 1-8("Kinde 2012")
1042	Meyerson et al. (2010) "Advances in understanding cancer genomes through second-generation sequencing," Nature Review Genetics, 11, 685-696 ("Meyerson")
1043	U.S. Patent No. 6,582,908 ("Fodor")
1044	Diehl et al. (2005) "Detection and quantification of mutations in the plasma of patients with colorectal tumors," Proc. Natl. Acad. Sci., 102(45), 16368-16373 ("Diehl")

1045	Declaration of Stephanie L. Schonewald
1046	Affidavit of Eric J. Marandett in Support of Petitioner's Motion for *Pro Hac Vice* Admission
1047	Affidavit of Sophie F. Wang in Support of Petitioner's Motion for *Pro Hac Vice* Admission
1048	Mishra & Whetstine (2016) "Different Facets of Copy Number Changes: Permanent, Transient, and Adaptive," Mol. Cell Biol. 36(7):1050-1063.
1049	Kinde et al. (2012) "FAST-SeqS: A Simple and Efficient Method for the Detection of Aneuploidy by Massively Parallel Sequencing," PLoS ONE, 7(7), 1-8, *and Supplemental Information*
1050	Foundation Medicine v. Guardant Health, IPR2019-00634, Transcript of Deposition of Carlos Bustamante, Ph.D., February 14, 2020
1051	Foundation Medicine v. Guardant Health, IPR2019-00634, Transcript of Deposition of John Quackenbush, Ph.D., February 19, 2020
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1053	Guardant Health v. Foundation Medicine, 1:17-cv-01616-LPS-CJB, Expert Report of Gregory Cooper, Ph.D. (Redacted)
1054	Guardant Health v. Foundation Medicine, 1:17-cv-01616-LPS-CJB, Transcript of Deposition of Gregory Cooper, Ph.D., October 15, 2019 (Redacted)
1055	U.S. Patent No. 10,388,403 ("Rava")
1056	PCT Publ. No. WO 2012/038839

1057	Sathirapongsasuti et al. (2011) "Exome sequencing-base copy-number variation and loss of heterozygosity detection: ExomeCNV," Bioinformatics, 27(19), 2648-2654
1058	Guardant Health v. Foundation Medicine, 1:17-cv-01616-LPS-CJB, Guardant's Opening Claim Construction Brief, D.I. 56 (Redacted)
1059	Guardant Health v. Foundation Medicine, 1:17-cv-01616-LPS-CJB, Report and Recommendation Regarding Claim Construction, D.I. 260
1060	Declaration of Dr. Stacey Gabriel In Support of Petitioner's Opposition to Patent Owner's Motion to Amend ("Second Gabriel Declaration")
1061	U.S. Pub. No. 2012/0046877 ("Hyland")
1062	Int. Pub. No. WO 2012/042374 ("Taipale")
1063	Deposition Transcript of John Quackenbush, Ph.D., Foundation Medicine, Inc. v. Guardant Health, Inc., Feb. 19, 2020

The Board should deny Patent Owner's Motion to Amend (Paper 26, "MTA") for U.S. Patent No. 9,840,743 ("'743 patent"). Patent Owner has not met its burden to show it is entitled to Substitute Claims 27-41 ("Substitute Claims") because it has failed to properly provide the requisite support in the original or earlier-filed disclosures. Moreover, even if Patent Owner had met its burden, the Substitute Claims are anticipated by or obvious over Rava (EX1055) or Hyland (EX1061).

I. PATENT OWNER FAILED TO MEET ITS BURDEN

Pursuant to 37 C.F.R. § 42.121(b), Patent Owner bears the burden in the Motion to Amend to set forth the support for each claim that is added or amended in the original or earlier-filed priority disclosures of the patent. As the Federal Circuit and the Board have held, "mere string citations without explanation are insufficient to meet [Patent Owner's] burden of production." *Intel Corp. v. Alacritech, Inc.*, IPR2017-01391, Paper 81 at 42-43 (PTAB Nov. 26, 2018) (chart providing string citations for each limitation without explanation is insufficient to provide written description support); *id.* at 44 ("[i]t is not the responsibility of the Board to search through the string citations to find sufficient written description support for each element…"); *see also B.E. Tech., L.L.C. v. Google, Inc.*, 2016 WL 6803057, at *7 (Fed. Cir. Nov. 17, 2016); *Lippert Components, Inc. v. Days Corp.*, IPR2018-00777, Paper 28 at 52 (PTAB Sept. 24, 2019).

Here, Patent Owner violates the Federal Circuit's clear directive by providing

little more than string citations as purported support for Substitute Claim 27 (with no explanation for how the cited passages might support the claim limitations), and ***only*** string citations in support of each of the proposed dependent claims. MTA, 6-15. Patent Owner's silence leaves open substantial questions about whether a person of ordinary skill in the art ("POSA") would have understood that the inventors had possession of the claimed method, particularly in view of the multiple, disparate portions of the '743 patent that are cited as purported support. *Compare* MTA, 7 (citing EX2028, ¶268, describing a method of detection using a "***single sample***," to support feature a)) *with id.*, 8 (citing EX2028, ¶281, describing a method of detection using a "***paired sample***," to support feature c)). Because Patent Owner has not provided a legally sufficient explanation for how the cited passages support the Substitute Claims, Patent Owner has not satisfied its burden of production under 37 C.F.R. § 42.121(b). For at least this reason, the Motion to Amend should be denied.

II. CLAIM CONSTRUCTION

A. "sub-chromosomal copy number variation"

Patent Owner's proposed construction of "sub-chromosomal copy number variation" ("CNV") as "a change in the copy number of a sub-chromosomal region" (MTA, 5) lacks support in the specification and only serves to create further ambiguity regarding the scope of the substitute claims. While Petitioner agrees that a "copy number variation" is a change in copy number, the rest of Patent Owner's

construction is unhelpfully circular; it uses, but does not define, "sub-chromosomal," and adds a new term ("sub-chromosomal region") not found in the '743 patent. *See Int'l Bus. Machines Corp. v. Intellectual Ventures II LLC.*, IPR2014-00660, Paper 58, 12 (PTAB Oct. 19, 2015) (rejecting construction that "adds no clarity").

Additionally, the '743 patent does not provide any definition or support for "sub-chromosomal copy number variation." Indeed, the phrase never appears in the '743 patent. *See generally* EX1001. As a result, Patent Owner attempts to piece together support from two passages in the specification—EX1001, 7:40-42 and 52:15-20.[1] MTA, 5. Patent Owner's attempt fails, however, because the first passage does not refer to sub-chromosomal CNVs, but rather "sub-chromosomal ***resolution***." EX1001, 7:41-45, *see also id.*, 19:56-60.[2] As explained by Patent Owner's own experts, resolution is a feature of a detection method, not a CNV. *See, e.g.*, EX1051, 104:13-14; EX1050, 107:15-18, 109:17-18. Thus, the passing description of "sub-chromosomal ***resolution***" does not provide the requisite support.

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[1] This post-hoc effort is both inadequate as a matter of claim construction and cannot serve to show that the "alleged inventor" possessed the purported invention as of the priority date. Rather, it again reflects Patent Owner's bare citations to disparate passages in the specification. *See Intel Corp.*, IPR2017-01391, Paper 81 at 43.

[2] Unless otherwise noted, all emphasis is added.

The second passage similarly fails, as it provides a list of genetic aberrations, including "copy number variations," without any size limitations or further description of the recited CNVs. Moreover, Patent Owner has adamantly represented that this passage describes "distinct genetic aberrations." POR, 17-18; EX2023, ¶¶22-23, 29. Thus, under Patent Owner's own view, other terms in this passage do not provide support for "***sub-chromosomal*** copy number variation."

For the above reasons, Patent Owner's construction should be rejected. For its analysis, Petitioner uses the customary and ordinary meaning of the separate terms "sub-chromosomal" (i.e., as "smaller than a chromosome") and CNV. *See, e.g.*, EX1051, 105:22-106:4, 106:5-15. Regardless of which construction the Board adopts, the Substitute Claims are unpatentable in view of the art cited herein.

B. "predefined region"

Patent Owner argues that a POSA would have understood "predefined region" to be "a region smaller than a whole chromosome." MTA, 5. As Petitioner explained, and as the Board accepted, "predefined region" is not so limited. *See, e.g.*, Pet. (Paper 2), 22, 27, 29; Institution Decision (Paper 10, "D.I."), 7-8 (citing *Johnson Worldwide Assocs. v. Zebco Corp.*, 175 F.3d 985, 991 (Fed. Cir. 1999)). Regardless, the Substitute Claims expressly limit the size of a "predefined region" as "up to 100kb." Thus, no construction in the Substitute Claims is necessary.

III. PRIOR ART

A. Rava (EX1055)

U.S. Patent No. 9,323,888 ("Rava"), entitled "Detecting and Classifying Copy Number Variation," was filed as U.S. Patent Application No. 13/600,043 on August 30, 2012. EX1055. Rava qualifies as prior art pursuant to 35 U.S.C. §102(a)(2).[3] Rava describes methods "for determining copy number variations (CNV) of a sequence of interest in a test sample that comprises a mixture of nucleic acids that are known or are suspected to differ in the amount of one or more sequence [sic] of interest." *Id.*, 2:31-35; EX1060, ¶ 19-23. "Sequences of interest include genomic segment sequences ranging from, e.g., kilobases (kb) to megabases (Mb) to entire chromosomes that are known or are suspected to be associated with a genetic or a disease condition." EX1055, 27:65-28:1; *see also id.*, 2:40-45; EX1060, ¶21.

Rava applies its methods to analyze nucleic acid molecules in test samples, such as "maternal sample[s]," which include "a mixture of fetal and maternal cell-free DNA molecules." EX1055, 9:8-10; EX1060, ¶22. Rava's methods "may employ next generation sequencing technology (NGS)" to obtain quantitative information, e.g., "each sequence read is a countable 'sequence tag' representing an individual clonal DNA template or a single DNA molecule." EX1055, 38:62-64, 39:2-; EX1002, ¶23. Rava uses generated sequencing data, *inter alia*, to:

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[3] The '743 patent is subject to the AIA's first-to-file provisions. EX1036, 4.

> align the reads to portions of a reference sequence/genome and determine the amount of DNA (e.g., the number of reads) that map to defined portions [of] the reference sequence (e.g., to defined chromosomes or chromosome segments); calculate a dose of one or more of the defined portions by normalizing the amount of DNA mapping to the defined portions with an amount of DNA mapping to one or more normalizing chromosomes or chromosome segments selected for the defined portion; determining whether the dose indicates that the defined portion is "affected[.]"

EX1055, 39:23-33; EX1002, ¶23.

B. Hyland (EX1061)

U.S. Publication No. 2012/0046877 ("Hyland"), entitled "Systems and Methods to Detect Copy Number Variation," published on February 23, 2012, and therefore is prior art to the '743 patent pursuant to 35 U.S.C. §102(a)(1). EX1061. Hyland describes, *inter alia*, methods "for copy number variation determination from analyzing biomolecule-related sequence reads." EX1061, Title, [0007]; EX1060, ¶¶24-29. Hyland's methods build off of well-known sequencing methods in 2012 and introduce then-novel data analyses for detecting CNVs. EX1061, [0006]-[0009]; EX1060, ¶26. Figure 3 of Hyland, e.g., shows a method, including (a) sequencing polynucleotides to generate sequence reads, (b) mapping the sequence reads to a reference genome, (c) quantifying the sequence reads in a plurality of windows, and (d) performing data analysis to detect copy number

variations. EX1061, Fig. 3, [0061]; EX1060, ¶27. In Figures 5 (single sample) and 7 (paired sample), Hyland provides additional details on the steps for quantifying reads and performing data analysis, including the performance of normalizing and data processing steps to detect CNVs. EX1061, Figs. 5 and 7; EX1060, ¶¶28-29.

IV. GROUNDS FOR UNPATENTABILITY

A. Ground 1: Rava Anticipates Substitute Claims 27 and 37-41

As shown below, Rava (particularly Example 20) discloses each and every element of Substitute Claims 27 and 37-41. Rava thus anticipates these claims.

1. Substitute Claim 27

A method for detecting sub-chromosomal copy number variation, comprising:

Rava discloses a method of detecting and classifying CNV, including specifically sub-chromosomal CNVs, e.g., "***deletions and/or duplications of segments of any one or more of the chromosomes***." *See, e.g.*, EX1055, Title, 2:31-48; *see also id.*, 27:65-28:5, 220:63-221:12 (referring to detecting "sub-chromosomal" variants). For instance, Example 20 discloses a method of noninvasively detecting fetal sub-chromosomal abnormalities, including, e.g., sub-chromosomal CNVs in Chromosomes 6 (sub-chromosomal duplication) and 7 (sub-chromosomal deletion), using NGS of maternal plasma. *Id.*, Example 20. Thus, to the extent the preamble is limiting, Rava discloses a method for detecting sub-chromosomal CNV. *See also* EX1060, ¶¶31-33.

a) sequencing extracellular polynucleotides from a bodily sample from a

subject, wherein each of the extracellular polynucleotides generates a plurality of sequence reads;

Rava discloses "sequencing cell free DNA from the maternal test sample to provide the sequence reads." EX1055, 12:5-34; *see also id.*, 10:44-11:14, 11:27-33, 12:10-17, 38:62-39:16, 39:22-23. Rava's methods include, *e.g.*, using NGS (*id.*, 32:32-35) and providing multiple sequence reads[4] wherein "each sequence read is a countable 'sequence tag' representing an individual clonal DNA template or a single DNA molecule" (*id.*, 39:3-5). In Example 20, Rava specifically discloses that "maternal plasma samples" in the "MatErnal BLood IS Source to Accurately Diagnose Fetal Aneuploidy ("MELISSA")" study library were sequenced "using TruSeq v3 chemistry on an Illumina HiSeq 2000 with single-end reads of 25 bp. In this example, each of the 11 maternal samples was sequenced utilizing an entire flow cell, resulting in $600x10^6$ to $1.3x10^9$ sequence [reads] per sample." *Id.*, 219:32-40.

Thus, Rava discloses Substitute Claim 27, step a). *See* EX1060, ¶¶34-39.

b) filtering out reads that fail to meet a set accuracy, quality score, or mapping score threshold;

Rava discloses "determining whether to include the read under consideration

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[4] Rava also discloses filtering out reads when multiple sequence reads map to the same "start and end coordinates" (EX1055, 166:63-64), indicating those sequences reads arose from clonal DNA templates or a single DNA molecule. EX1060, ¶36.

in the number of sequence tags for a chromosome of interest or a chromosome segment of interest." EX1055, 11:15-26. Rava states that "[v]arious criteria may be set for choosing when to disregard an identical tag from a single sample." *Id.*, 51:29-50. One exemplary criteria for filtering reads is an allowable limit in the "amount of mismatch in aligning to a reference genome." *Id.*, 33:36-51; *see also id.*, 34:5-20, 50:58-62. "Tags that can be mapped to more than one location on a reference genome i.e., tags that do not map uniquely," can be filtered out prior to analysis. *Id.*, 33:49-51. In Example 20, Rava specifically describes that "only reads that unambiguously mapped to a single genomic location" with "up to two base mismatches during alignment" were allowed to be used in the read count analysis. *Id.*, 220:31-38. Because Rava discloses excluding reads based on a mapping score threshold, Rava discloses Substitute Claim 27, step b). *See also* EX1060, ¶¶40-42.

c) mapping the plurality of sequence reads to a reference sequence;

Rava discloses "aligning the sequence reads to one or more chromosome reference sequences using a computing apparatus and thereby providing sequence tags corresponding to the sequence reads; … ." EX1055, 10:44-11:14; *see also id.*, 12:5-34, 16:44-20:14, 39:17-36. Rava also clarifies that this aligning step refers to:

> the process of comparing a read or tag to a reference sequence and thereby determining whether the reference sequence contains the read sequence. If the reference sequence contains the read, the read may be mapped to the reference sequence or, in certain embodiments, to a

particular location in the reference sequence.

Id., 33:52-34:4. Example 20 specifically states, "[s]equence reads were aligned to the human genome assembly hg19 obtained from the UCSC database [ ]. *Id.*, 220:31-43. Rava thus discloses Substitute Claim 27, step c). *See* EX1060, ¶¶46-49.

d) quantifying mapped reads or unique sequence reads in a plurality of predefined regions of the reference sequence; and

Rava discloses "determin[ing] the amount of DNA (e.g., the number of reads) that map to defined portions the reference sequence (e.g., to defined chromosomes or chromosome segments)…." EX1055, 39:17-36. Rava further discloses that the sequence reads that are quantified can be unique sequence reads[5]: "The software reads the sequence data generated from the above procedure that uniquely aligned to the genome from Bowtie output (bowtieout.txt files). Sequence alignments with up to 2 base mis-matches were allowed and included in alignment counts only if they aligned ***uniquely*** to the genome. *Id.*, 166:58-167:5; *see also id.*, 174:45-46. Rava

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[5] As set forth in the Petition and Reply to Patent Owner's Response (Paper 33), a POSA would have understood that "unique sequence reads" are "sequence reads that can be mapped to just one location," and can be determined to correspond to a particular extracellular polynucleotide. *See, e.g.*, Pet., 24; Reply, 14; *see also* EX1060, ¶47.

exemplifies this step in Example 20, where "reads that unambiguously mapped to a single genomic location were included," and "[g]enomic sites at which [those] reads mapped were counted as tags." *Id.*, 220:36-38. Thus, Rava discloses Substitute Claim 27, step d). *See also* EX1060, ¶¶34-39.

e) determining sub-chromosomal copy number variation in one or more of the plurality of predefined regions by:

Rava discloses determining sub-chromosomal CNVs utilizing both the "normalizing[6]" and "processing" steps of Claim 27, step e). *See, e.g.*, EX1055, 221:41-54 ("[d]etection of a sub-chromosomal abnormality was a multi-step process for classifying specific regions as having a copy number variant.").

i) normalizing a number of reads in the plurality of predefined regions to each other, or a number of unique sequence reads in the plurality of predefined regions to each other; and/or

Rava discloses normalizing a number of reads (e.g., unique sequence reads) in a plurality of bins (i.e., predefined regions[7]) to each other. As discussed above,

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[6] The '743 does not define "normalization"; however, "normalization" generally "refers to methods of adjusting data that allow for comparison of data from different sources." EX1002, ¶90; *see also* EX1050, 28:3-6; EX1060, ¶51.

[7] Patent Owner has conceded that "bins" or "windows" are the same as "predefined regions" in the context of the '743 patent. *See, e.g.*, MTA, 9.

Rava quantifies unique sequence reads. *Id.*, 166:58-167:5, 220:36-38. Rava normalizes the read counts in its bins by calculating a Bin Ratio Value ("BRV") for each bin, where the BRV equals the ratio of tags within a bin to the sum of the number of tags in certain other bins in the assay, i.e., a select number of bins with the nearest GC percentages. *Id.*, 220:44-56; *see also id.*, 220:58-62 (describing that the sum "over 40 bins" is used "for the 100 kb data for bins ([km]) with the nearest GC percentage to bin ij"). As Patent Owner's expert, Dr. Bustamante, admitted, this comparison is a type of normalization specifically contemplated by the '743 patent. *See* EX1050, 28:3-6 ("The normalizing step can encompass multiple types of analysis where one is taking the -- the reads in the plurality predefined regions and analyzing them as an ensemble."), 151:11-19 (agreeing that a "comparison" of tagged nucleotides is "normalization"); *see also* MTA, 9 (citing EX2028, ¶221 as supporting the "normalizing" step, which includes comparing "the amount of tagged parent molecules" mapping to a first and second "location or mappable position in a reference sequence"). Thus, Rava discloses Substitute Claim 27, step e)ii). *See* EX1060, ¶¶50-55.

ii) processing a number of reads in the plurality of predefined regions or a number of unique sequence reads in the plurality of predefined regions with numbers obtained from a control sample,

Rava discloses processing a number of reads (e.g., unique sequence reads) in a plurality of bins (i.e., predefined regions) with numbers obtained from a control

sample. Rava describes using, e.g., a "qualified sample," which is "a sample comprising a mixture of nucleic acids that are present in a known copy number to which the nucleic acids in a test sample are to be compared, and it is a sample that is normal i.e. not aneuploid, for the sequence of interest." EX1055, 30:62-31:7.

Example 20 of Rava discloses that "[i]n order to detect any sub-chromosomal differences, each of the [Bin Ratio Values] were examined for deviations from ***the median values measured across multiple samples***," which included artificial samples having known copy number statuses. *Id.*, 220:63-221:12; *see also id.*, 218:50-61. This comparison in Rava—*i.e.*, the Bin Ratio Value of a bin with the median values across samples—discloses Substitute Claim 27's "processing" step e)ii). EX1060, ¶57. Other steps in Rava also disclose this limitation.

For instance, Rava uses median values in a series of calculations to ultimately "calculate a z-score for each bin." *See* EX1055, 220:58-221:12. Because a "z-score" refers to the number of standard deviations from ***an average value of a reference data set*** (*see, e.g.*, EX1022, 20459; EX1002, ¶96, EX1060, ¶58)—here, median values—a POSA would have understood that the calculation of z-scores includes processing the sequence reads in each bin with a control, per step e)ii). EX1060, ¶58; *see also* EX1002, ¶¶94-95. As explained by Rava, the z-scores can be compared to a control to determine the presence of CNVs: "It was expected that z_{ij} would be approximately ±3 for regions without any copy number variations (CNVs)

and significantly greater than 3 when fetal CNVs were present." EX1055, 221:13-15.[8] Thus, Rava discloses Substitute Claim 27, step e)ii). *See* EX1060, ¶¶56-60.

wherein each of the predefined regions is up to 100 kb.

Rava discloses that "determination of a CNV of a chromosome segment is performed by first subdividing the chromosome of interest into sections or bins of variable length," where the "bin length can be of ***at least about 1 kbp***, ***at least about 10 kbp***, ***at least about 100 kbp***, at least about 1 mbp, at least about 10 mbp, or at least about 100 mbp." EX1055, 49:60-67. Example 20 describes dividing the genome "into 1 Mb and ***100 kb bins***…." *Id.*, 220:44-56. For these reasons, Rava anticipates Substitute Claim 27. *See* EX1060, ¶¶61-62.

2. Substitute Claim 37-41

Substitute Claim 37 recites that the method comprises both steps e)i) and e)ii). Substitute Claims 38 and 39 recite that step e) comprises i) normalizing the number of reads or unique sequence reads, respectively, in the plurality of predefined regions to each other; and Substitute Claims 40 and 41 recite that step e) comprises ii)

---

[8] In turn, z-scores can be "utilized to determine the relative fetal fraction (ff) present in the cfDNA. ***The value can then be compared to an independent measurement of ff to validate copy number detection***, or suggest the presence of mosaicism." EX1055, 221:16-19; *see also* EX1060, ¶59.

processing the number of reads or unique sequence reads, respectively, in the plurality of predefined regions with numbers obtained from the control sample. As explained above, Rava anticipates each limitation of Substitute Claim 27, including steps e)i) and e)ii) using a number of unique sequence reads, and thus a number of reads. *See* Section IV.A.1. Accordingly, Rava also anticipates claims 37-41. *See* EX1060, ¶¶64-65.

B. Ground 2: Substitute Claims 28-36 Are Obvious Over Rava

Substitute Claims 28-36 all depend from Substitute Claim 27. As discussed above, Rava anticipates Substitute Claim 27. Rava further teaches the features of Substitute Claims 28-29 and 31-35, as explained below.

1. Substitute Claim 28

Substitute Claim 28 recites that the method further comprises "isolating extracellular polynucleotides from the bodily sample." MTA, 23. Rava teaches that "[t]o separate cell-free DNA from cells in a sample, various methods including, but not limited to fractionation, centrifugation (e.g., density gradient centrifugation), DNA-specific precipitation, or high-throughput cell sorting and/or other separation methods can be used." EX1055, 54:2-7. A POSA would have understood that, applying Rava's teachings "[t]o separate cell-free DNA from cells in a sample" to maternal plasma samples, which include cells and cell-free DNA, would isolate cell-free DNA from a bodily sample. EX1060, ¶67. As such, Rava renders Substitute

Claim 28 obvious. *See id.*, ¶68; *see also* EX1063, 53:15-17; EX1001, 30:27-36.

2. Substitute Claim 29

Rava teaches that "[i]n various embodiments the cfDNA present in the sample can be enriched specifically or non-specifically ***prior to use*** (e.g., ***prior to preparing a sequencing library***)." EX1055, 54:15-17. Rava explains that nonspecific enrichment of sample DNA can refer, *e.g.*, "to the whole genome ***amplification*** of the genomic DNA fragments of the sample that can be used to increase the level of the sample DNA ***prior to preparing a cfDNA sequencing library***." *Id.*, 54:17-42. Rava thus renders Substitute Claim 29, wherein the method comprises "generating copies of the extracellular polynucleotides prior to sequencing," obvious. *See* EX1060, ¶¶69-71.

3. Substitute Claim 30

Rava teaches that its method can be used to calculate the percent of sequences having a short tandem repeat (STR) variant:

> The fraction of the minor allele for any given informative marker is calculated by dividing the peak height of the minor component by the sum of the peak height for the major component, and ***the fraction is expressed as a percent for each informative locus*** as
>
> $$(\%\ \text{fetal fraction}) = \left[\frac{\text{peak height of minor allele(s)}}{\sum(\text{peak height of major allele(s)})}\right] \times 100 \quad \text{Equation 3}$$

EX1055, 134:40-50. Thus, Rava renders obvious the additional limitation of Substitute Claim 30, "determining a percent of sequences having copy number

variation or rare mutation or variant in the bodily sample." *See* EX1060, ¶¶72-74.

4. Substitute Claims 31-33

Rava renders Substitute Claims 31, 32, and 33 obvious. Substitute Claim 31 recites that the method further comprises "attaching one or more barcodes to the extracellular polynucleotides or fragments thereof prior to sequencing." MTA, 23. Substitute Claim 32 states that "each barcode attached to the extracellular polynucleotides or fragments thereof prior to sequencing is not unique." *Id.*, 24. Substitute Claim 33 recites that "each barcode comprises a fixed or semi-random oligonucleotide sequence that in combination with a diversity of molecules sequenced from a selected region enables identification of unique molecules." *Id.*

Rava discloses using NGS to sequence, e.g., DNA from multiple samples, which "can be pooled and sequenced as indexed genomic molecules (i.e., multiplex sequencing) on a single sequencing run, to generate up to several hundred million reads of DNA sequences." EX1055, 39:9-14. "Parallelizing sequencing for multiple samples i.e. multiplex sequencing, requires the incorporation of ***sample-specific index sequences, also known as barcodes***, during the ***preparation of sequencing libraries***." *Id.*, 77:14-17. Rava teaches that one application of the index sequences is to verify the integrity of the multiple samples, which is "accomplished by first grouping sequence tags associated with ***identical index sequences*** to associate the genomic and marker sequences and ***distinguish sequences belonging to each of the***

libraries made from genomic molecules of a plurality of samples." *Id.*, 78:9-14. Because Rava discloses adding "sample-specific" barcodes prior to sequencing in order to later distinguish sequences from each sample, a POSA would have understood that extracellular polynucleotides in a sample would have a common (i.e., non-unique) index (i.e., barcode). EX1060, ¶¶76-77.

Moreover, Rava describes that the sample-specific barcodes can be incorporated into PCR primers used for amplification. EX1055, 77:14-25. A POSA would have understood that a "sample specific" barcode sequence would be known or "fixed" prior to adding the barcode to polynucleotides. EX1060, ¶78. Also, because unique molecules in a multiplex sample can be identified by, (1) mapping sequence reads to determine their start/end coordinates (*see, e.g.*, EX1055, 166:58-167:5), and (2) attributing different reads with the same start/end coordinates to particular samples using sample-specific barcodes (*id.*, 77:14-17), a POSA would have understood that Rava's fixed sample-specific barcodes in combination with the sequenced molecules enable identification of unique molecules. Thus, Rava renders Substitute Claims 31-33 obvious. *See* EX1060, ¶¶78-79; *see also* EX1001, 37:50-59.

5. Substitute Claim 34

Rava discloses that "[i]n various embodiments the cfDNA present in the sample can be enriched specifically or non-specifically ***prior to use*** (e.g., ***prior to***

preparing a sequencing library)." EX1055, 54:15-17. For example, Rava describes a method comprising "***enriching*** the mixture of nucleic acids for polymorphic target nucleic acids in operation" and "***sequencing the enriched mixture*** of nucleic acids in operation." *Id.*, 128:35-42; *see also*, *e.g.*, *id.*, 131:7-12 ("[i]n other embodiments, the cfDNA in the sample is enriched specifically. Specific enrichment refers to the enrichment of a genomic sample for specific sequences, e.g. polymorphic target sequence … ."); 54:21-27, 128:57-67, 129:1-25, 129:32-37. Rava thus teaches Substitute Claim 34, "selectively enriching regions from a genome or transcriptome of the subject prior to sequencing." *See* MTA, 24; EX1060, ¶¶80-82.

6. Substitute Claim 35

Substitute Claim 35 recites that the method further comprises "attaching one or more barcodes to the extracellular polynucleotides or fragments thereof prior to an amplification or enrichment step." MTA, 24. Rava teaches, e.g., that "[a]lternatively, the index sequence can be incorporated into the adaptor, which is ligated to the cfDNA prior to the PCR amplification." EX1055, 77:25-28. In view of this teaching, Rava renders Substitute Claim 35 obvious. *See* EX1060, ¶¶83-84.

7. Substitute Claim 36

Rava teaches identifying single nucleotide polymorphisms (SNPs) in fetal and maternal DNA. *See, e.g.*, EX1055, 22:54-59, 129:51-130:24, Fig. 12. Rava also teaches that smaller bin sizes are desirable for detecting small mutations, like SNPs.

For example, Rava states: "The smaller the bin length, the greater the resolution that is obtained to localize the CNV of the segment in the chromosome of interest." *Id.*, 49:65-67. A POSA reading Rava would have understood that adjusting the bin length (i.e., using a smaller predefined region) readily could be performed to allow for resolution or detection of a small mutation. EX1060, ¶¶85-86. Specifically, a POSA would have understood that single-base predefined regions could be used in order to resolve or detect a single base mutation, such as a SNP. Thus, the added limitation of Substitute Claim 36 ("wherein each of the plurality of predefined regions is a single base") fails to distinguish this claim over Rava. *See* MTA, 24-25.

Moreover, as of September 4, 2012, it was well known in the art that single-base mutations were among the most common type of mutations in cancer and other diseases. EX1002, ¶160; EX1042, 690-91 ("[n]ucleotide substitution mutations are the most common known somatic genomic alteration in cancer" and NGS methods "offer substantial benefits for copy number analysis, including higher resolution (up to the level of the single-base insertion or deletion)"); EX1061, [0027]. Given that single-base mutations were among the most common type of mutations in cancer and other diseases, and that Rava teaches detecting small mutations (e.g., SNPs) with smaller predefined regions, it would have been obvious to a POSA to analyze polynucleotides from a bodily sample from a subject by using a plurality of regions each consisting of a single base. EX1060, ¶87. Further, as Rava teaches identifying

SNPs using its methods, a POSA would have had a reasonable expectation that predefined regions of a single base could be successfully used. *Id.* Rava thus renders Substitute Claim 36 obvious. *See id.*, ¶¶85-88.

C. Ground 3: Substitute Claims 27 and 38-41 Are Obvious in View of Hyland

1. Substitute Claim 27

A method for detecting sub-chromosomal copy number variation, comprising:

Hyland teaches "Systems and Methods to Detect Copy Number Variation." EX1061, Title; *see also id.*, [0027], Figs. 3, 5, and 7. Hyland describes analyzing data polynucleotide sequencing and mapping the generated sequence reads to determine CNVs. *See, e.g., id.*, [0007], Fig. 5, Fig. 7, Claim 1, 11. Hyland explains that its methodology can be used for determining CNVs that are smaller than a chromosome, illustrating the results of CNV detection within a portion of Chromosome 11. *Id.*, Fig. 8A; *see also, e.g., id.*, [0067], [0107], Figs. 6A and 6B. Thus, Hyland discloses the preamble of Substitute Claim 27. *See* EX1060, ¶¶89-90.

a) sequencing extracellular polynucleotides from a bodily sample from a subject, wherein each of the extracellular polynucleotides generates a plurality of sequence reads;

Hyland describes "sequenc[ing] a plurality of nucleic acid fragments obtained from a single biological sample and generat[ing] a data file containing a plurality of fragment sequence reads that are representative of the genomic profile of the biological sample." EX1061, [0064]; *see also id.*, [0007], [0027], [0053], [0059],

and Figs. 2, 3, 5, and 7. While Hyland does not expressly disclose that the sequenced genomic DNA (*see, e.g., id.*, [0061], Fig. 3) is extracellular, a POSA would have understood that Hyland's sequencing step could be used with extracellular polynucleotides with a reasonable expectation of success. As Dr. Bustamante acknowledged, "[w]hether or not polynucleotides are extracellular or not, the sequencing machine doesn't care. It will generate sequence data." EX1050, 132:23-25. Indeed, in 2012, it was common knowledge that extracellular polynucleotides could be sequenced and analyzed for genetic variants, including CNVs. *See, e.g.*, EX1060, ¶92; EX1022, Fig. 1; EX1026; EX1033; EX1035; EX1041; EX1044; EX1062, Example 4. Thus, Substitute Claim 27, step a) would have been obvious in view of Hyland. *See* EX1060, ¶¶91-93.

b) filtering out reads that fail to meet a set accuracy, quality score, or mapping score threshold;

Hyland teaches that sequence reads in window regions can be filtered before merging windows into a segment based on "minimum segment length requirements or ***window region mappability thresholds***, etc." EX1061, [0099]; *see also id.*, [0065], [0086], [0110]. To assess mappability, Hyland's "mappability files have one row per every position, indicating whether each position is or is not uniquely mappable." *Id.*, [0070]. Accordingly, Hyland teaches Substitute Claim 27, step b). *See* EX1060, ¶¶94-95.

c) mapping the plurality of sequence reads to a reference sequence;

Hyland teaches that "genomic fragment reads are mapped to a reference genome (i.e., template genome)." EX1061, [0061]. To perform this step, Hyland teaches, e.g., that a "[m]apping engine 405 can be configured to receive nucleic acid (fragment) sequence read data output from nucleic acid sequencer 403, [and] ***map the reads to a reference genome***…." *Id.*, [0067]. Thus, Hyland teaches Substitute Claim 27, step c). *See* EX1060, ¶96.

d) quantifying mapped reads or unique sequence reads in a plurality of predefined regions of the reference sequence; and

Following mapping, Hyland teaches that "the chromosomal regions are divided into variable-sized genomic windows and read coverage is determined for each window." EX1061, [0061]; *see also id.*, [0067], [0092], [0094], [0104], and [0106]. Hyland describes, e.g., that a read coverage engine can be configured to

> divide each of the chromosomal regions into one or more non-overlapping variable size window regions, ***determine sequence read coverage for each of the variable sized window regions*** and normalize the sequence read coverage determined for each window region to correct for bias (such as GC bias).

Id., [0069]. Thus, Hyland teaches Substitute Claim 27, step d). *See* EX1060, ¶97.

e) determining sub-chromosomal copy number variation in one or more of the plurality of predefined regions by:

To detect CNVs, Hyland teaches using both steps e)i) and e)ii).

i) normalizing a number of reads in the plurality of predefined regions to each other, or a number of unique sequence reads in the plurality of predefined

regions to each other; and/or

Hyland teaches normalization of aligned (i.e., mapped) sequence reads using a "normalization engine," which "can be configured to divide each chromosomal region into one or more non-overlapping window regions, determine nucleic acid sequence read coverage for each window region and normalize the nucleic acid sequence read coverage determined for each window region to correct for bias." EX1061, Abstract; *see also id.*, [0078], [0080], [0085], [0095], [0098]-[0099], [0061] ("For coverage normalization, the variable-sized genomic windows are selected to contain a constant number of mappable positions (such an approach can smooth stochastic sampling noise)."). *Compare* MTA, 9 (citing EX2034, ¶58 as support for Substitute Claim 27, step e)i)). Hyland teaches that in some embodiments, "each of the window regions are sized so that they contain about the same number of uniquely mappable bases." EX1061, [0070]. A POSA would understand that normalization may be carried out in regions with uniquely mappable bases, i.e., with unique sequence reads. EX1060, ¶101. Thus, Hyland also teaches Substitute Claim 27, step e)i). *See id.*, ¶¶98-101.

ii) processing a number of reads in the plurality of predefined regions or a number of unique sequence reads in the plurality of predefined regions with numbers obtained from a control sample,

Hyland teaches processing reads (e.g., normalizing reads) with numbers (of reads) from a control sample. For example, Hyland discloses:

> Paired sample CNV detection shares many of the sample steps/operations as those used in single sample CNV detection. However, as depicted in FIGS. 6A and 6B, in the case of paired-sample CNV detection, … the coverage of the test sample can be normalized by comparing it to the coverage of a control sample.

EX1061, [0101]; *see also id.*, [0107]-[0110]. *Compare* MTA, 10 (citing EX2034, ¶66). As described above, a POSA would understand that a window region may include unique reads. *See supra* p. 24. Thus, Hyland teaches step e)ii). *See* EX1060, ¶¶102-104.

wherein each of the predefined regions is up to 100 kb.

Hyland discloses that "each window region contains about 5000 mappable bases." EX1061, Claims 3 and 16. Thus, Hyland teaches a method in which each of the predefined regions is up to 100 kb. *See* EX1060, ¶105.

For these reasons, Substitute Claim 27 is obvious in view of Hyland.

2. Substitute Claim 38-41

As explained above, Hyland discloses each limitation of Substitute Claim 27, including steps e)i) normalizing and e)ii) processing using a number of unique sequence reads, and thus a number of reads. *See* Section IV.C.1. Accordingly, Hyland also anticipates Claims 38-41, which recite the normalizing and processing steps using a number of reads or unique sequence reads. *See* EX1060, ¶107.

V. CONCLUSION

For these reasons, Patent Owner's Motion to Amend should be denied.

Respectfully submitted,

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The undersigned hereby certifies that the foregoing **PETITIONER'S OPPOSITION TO PATENT OWNER'S MOTION TO AMEND** was served electronically in its entirety on March 3, 2020 via electronic mail to the following attorneys of record:

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

FOUNDATION MEDICINE, INC.,
Petitioner,

v.

GUARDANT HEALTH, INC.,
Patent Owner.

Case No. IPR2019-00634
U.S. Patent No. 9,840,743

PETITIONER'S REPLY TO PATENT OWNER RESPONSE

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EXHIBIT LIST

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1001	U.S. Patent No. 9,840,743 ("the '743 patent")
1002	Declaration of Dr. Stacey Gabriel ("Gabriel Declaration")
1003	*Curriculum Vitae* of Dr. Stacey Gabriel
1004	U.S. Patent Application No. 15/467,570, March 23, 2017, Certification and Request for Prioritized Examination Under 37 C.F.R. § 1.102(e)
1005	U.S. Patent Application No. 15/467,570, April 27, 2017 Decision Granting Request for Prioritized Examination
1006	U.S. Patent Application No. 15/467,570, Original Claims as filed
1007	U.S. Patent Application No. 15/467,570, April 20, 2017, Preliminary Amendment
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1009	U.S. Patent Application No. 15/467,570, June 1, 2017, First Action Interview Pilot Program Pre-Interview Communication/Office Action
1010	U.S. Patent Application No. 15/467,570, June 29, 2017, Response to Pre-Interview First Office Action
1011	U.S. Patent Application No. 15/467,570, July 13, 2017, Applicant-Initiated Interview Summary
1012	U.S. Patent Application No. 15/467,570, August 4, 2017, Notice of Allowance

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1062	Int. Pub. No. WO 2012/042374 ("Taipale")

I. INTRODUCTION

Patent Owner's Response (Paper 25, "POR") relies principally on improper claim constructions that are unsupported by the intrinsic record and contradicted by Patent Owner's own statements and its experts' admissions. The Board correctly found in its Institution Decision (Paper 10, "D.I.") that, in the context of the '743 patent, "copy number variation" includes aneuploidy, and "predefined regions" is "not limited to any specific number of base pairs, but encompasses any predefined region of a genome, including an entire chromosome." D.I., 8, 10. As discussed herein and in the Petition, Patent Owner's arguments on Grounds 1 and 2 fail.

For Grounds 3 and 4, the Board mistakenly relied on Patent Owner's arguments regarding "unique sequence reads" in providing *sua sponte* a construction of "unique sequence reads" that excludes the use of barcodes, which neither party advocated. D.I., 11-12, 31-32. Based on its preliminary construction, the Board found that Kinde 2011 and Forshew teach "the opposite of a unique sequence read." *Id.*, 31. But the specification and the claims do not support this construction, as Patent Owner's expert admitted. Indeed, Patent Owner itself does not even appear to adopt the Board's construction. *Compare* POR, 46-47 *with* Patent Owner's Preliminary Response (Paper 6, "POPR"), 27-28. Because the Petition correctly applied the ordinary and customary meaning of "unique

sequence reads," the challenged claims also are unpatentable on Grounds 3 and 4.

II. PATENT OWNER'S CLAIM CONSTRUCTIONS ARE FLAWED

The '743 patent claim terms should be given their ordinary and customary meaning as understood by a person of ordinary skill in the art ("POSA")[1] in 2012. *See* Petition, 16-17; D.I., 6-7; *Phillips v. AWH Corp.*, 415 F.3d 1303, 1313-14 (Fed. Cir. 2005) (en banc).

A. "copy number variation"

Patent Owner's central defense of the challenged claims rests on its construction that "copy number variation" ("CNV") excludes aneuploidy. POR, 21. This construction is contrary to the '743 patent, Patent Owner's experts' admissions, Patent Owner's own statements in this proceeding and in litigation, and the understanding in the art.

First, Patent Owner incorrectly asserts that the specification describes CNV and aneuploidy as "distinct[2] genetic aberrations." POPR, 21; POR, 18. The Board

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[1] While Patent Owner criticizes Dr. Gabriel's definition of a POSA, it offers no alternative definition, and, in fact, adds more requirements to the definition. *See* EX2026, ¶¶22-24; EX1051, 16:11-15; EX1050, 15:3-16:10, 17:15-20.

[2] Notably, Patent Owner's expert, Dr. Quackenbush, repeatedly resisted taking a position on whether CNV "excludes" aneuploidy. *E.g.*, EX1051, 59:17-66:10 ("I

correctly found at institution that the intrinsic record does not support Patent Owner's narrow definition of CNV. D.I., 10. For example, Patent Owner relies heavily on the specification's listing of "CNV" and "aneuploidy" among other terms. POPR, 21; POR, 18-19 (citing EX1001, 52:15-20). Yet, as the Board noted, a complete reading of the specification shows that many listed terms are synonymous with, or subsets of, each other. D.I., 9-11. For example, the specification describes detecting "mutations, ***rare mutations***, ***indels***, copy number variations, … deletions, aneuploidy, partial aneuploidy, … ***abnormal changes in epigenetic patterns, abnormal changes in nucleic acid methylation***…." EX1001, 52:15-26.[3] *See also id.*, 2:65-3:9, 3:66-4:8, 7:5-15. The specification expressly equates these terms with each other. *E.g.*, *id.*, 30:51-54 (describing "rare mutations (***e.g.,*** single or multiple nucleotide variations) or changes in epigenetic markers, ***including*** but not limited to methylation profiles"), 1:21-28, 2:65-3:9, 15:8-21. Thus, the specification does not support Patent Owner's assertion that the listed

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don't use the term '***include'*** or '***exclude***.' I would say it is ***distinct*** from aneuploidy…"). Instead, he equivocated, acknowledging that "distinct" words could refer to subsets of each other, but admitting that he did not analyze whether the "distinct" term CNV could include aneuploidy. *Id.*, 50:22-51:20, 63:11-16.

[3] Unless otherwise noted, all emphasis is added.

terms must be mutually exclusive.

Similarly, Patent Owner's reliance on the specification's purported description of CNVs "at the gene level" is misplaced. POR, 19, 26-27. The cited passages refer only to embodiments, which, as discussed below, do not actually limit CNVs in any way. Moreover, even assuming that the specification describes a "sub-chromosomal" CNV, limiting "CNV" to only gene-level aberrations would fall into the "the danger of reading limitations from the specification into the claim." *Phillips*, 415 F.3d at 1323. *See Cont'l Circuits LLC v. Intel Corp.*, 915 F.3d 788, 797 (Fed. Cir. 2019).

In fact, contrary to Patent Owner's contentions (POR, 18, 34), the specification at most teaches an embodiment of detecting CNVs by using "sub-chromosomal ***resolution***."[4] EX1001, 7:38-45, 19:55-57. This embodiment thus merely provides one[5] example of the resolution of a disclosed method, not a

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[4] "Sub-chromosomal" only appears twice in the specification, in nearly identical passages, and only in the context of resolution. EX1001, 7:38-45, 19:55-57.

[5] Even if the patent only described a single embodiment—which is not the case here—the Federal Circuit has repeatedly "'rejected the contention" that "the claims of the patent must be construed as being limited to that embodiment.'" *Cont'l*

definition of "CNV" that limits CNV to any particular size. *See* EX1051, 102:3-21, 104:13-16 ("So the resolution is the size in which we can see things, so we're provided different resolution limits and those resolution limits range from 100 megabases[6] down to 1 kilobase."). Thus, the specification does not limit "CNV" to exclude aneuploidy. *See Phillips*, 415 F.3d at 1323.

Indeed, the Board properly recognized that Patent Owner itself has equated the purportedly "distinct" terms listed in the '743 patent with each other. POPR, 16 ("Claims 1 and 10…are directed to methods for detecting rare mutation, ***such as copy number variation***…."); D.I., 10. Patent Owner's experts also have repeatedly agreed that the listed terms describe overlapping categories. *E.g.*, EX1050, 20:6-13 ("indel" is "a type of mutation" and "could be a rare mutation"), 21:10-12 ("The word 'indel' describes a series of potential kinds of changes which could include insertions, and it could include deletions."), 24:3-4 ("we often refer to methylation as a type of epigenetic change"), 25:23-24 ("There are cases in

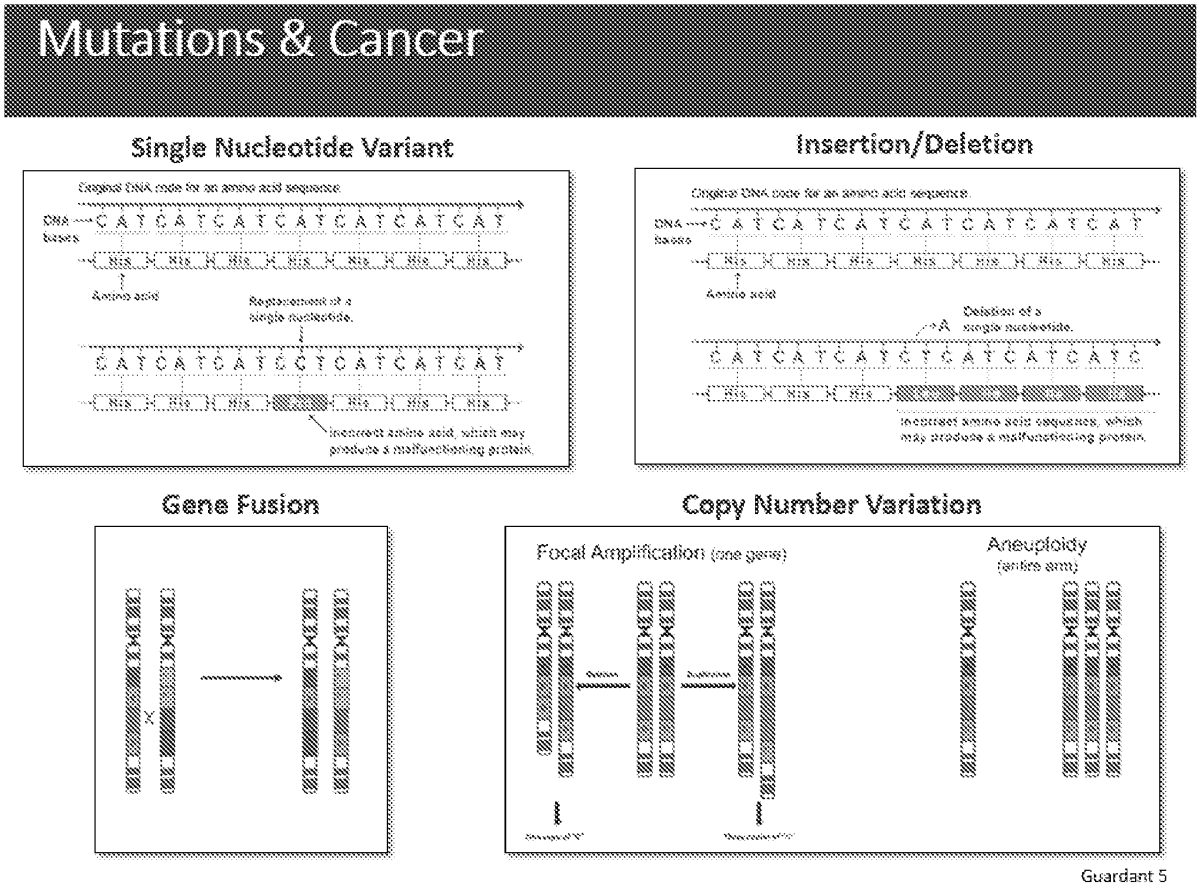
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Circuits, 915 F.3d at 797 (quoting *Phillips*, 415 F.3d at 1323); *Liebel-Flarsheim Co. v. Medrad, Inc.*, 358 F.3d 898, 906 (Fed. Cir. 2004).

[6] Moreover, even if CNV were limited to "100 megabases," some chromosomes are actually much smaller than 100 Mb. *See* POR 13-14 (human chromosome 21, "the subject chromosome of Chiu and Kinde 2012," is about 46 Mb).

which the deletion of a chromosome would be described as an aneuploidy."), 43:3-7 (CNVs "can be rare"); EX1051, 40:5-6 ("[s]ingle nucleotide variants" "might be common or rare"). In fact, Patent Owner admits that the specification's "recitation of mutations and rare mutations ***may include the other listed variations***," yet contends, without support, that the same logic does not apply to CNV and aneuploidy. POR, 18. *Cf. Eli Lilly & Co. v. Teva Parenteral Meds., Inc.*, 845 F.3d 1357, 1371-72 (Fed. Cir. 2017) (listing of classes within a recited group was overlapping); *Multilayer Stretch Cling Film Holdings, Inc. v. Berry Plastics Corp.*, 831 F.3d 1350, 1364 (Fed. Cir. 2016) (same).

Patent Owner's assertions are directly contradicted by its own representations in the parallel proceeding, *Guardant Health, Inc. v. Foundation Medicine, Inc.*, 1:17-cv-01616-LPS-CJB (D. Del.), that aneuploidy is a type of CNV, and that "Guardant's technologies provide methods of detecting ***all of these mutations***." EX1052, 5. Patent Owner provided a tutorial to the District Court that included a figure depicting "Aneuploidy" under CNV (reproduced below):



EX1052, 5. On the slide, Patent Owner explained:

> There are four basic types of genetic mutations that lead to cancer, shown in the four figures presented. … Finally, ***in the lower right, there are copy number variations, also known as gene amplification, where whole genes or even entire chromosomes are duplicated within a cell. In the figure, two examples are shown***. First, there is a copy number variation in which a single gene is either deleted or duplicated. As an example, for the blue gene labeled C, there can be one less copy or one extra copy. ***Another type of copy number variation is when an entire chromosome is duplicated or deleted.*** While normal cells have two copies of each chromosome, these

> mutations create situations where a cell has only one chromosome or an extra copy of the chromosome.

Id. Patent Owner's litigation expert, Dr. Gregory Cooper, likewise admitted that CNVs include aneuploidy. EX1053, ¶37; EX1054, 408:25-409:5 ("Q. And trisomy-21 is a form of copy number variation? A. It's an aneuploidy. Yes. Q. Which is a form of a copy number variation; correct? A. Yes."), 30:2-3 ("Like I said, I think an aneuploidy is a special subset of CNVs.").

Patent Owner's construction is also inconsistent with the ordinary and customary use of CNV in the art. *See* D.I., 10 (citing EX1026, 1048 ("Because we are collecting information across the entire genome, this method is capable of detecting ***any aneuploidy or other copy number variation*** including insertions and deletions.")). *See also* EX1055, 2:40-46 ("***CNV*** that can be determined according to the present method ***include trisomies and monosomies of any one or more of chromosomes*** 1-22, X and Y...."); EX1056, 24:9-12 ("***Copy number variations can include*** genomic rearrangements such as deletions, duplications, inversions, and translocations or ***whole chromosome aneuploidies***...."); EX1029, 189; EX1028, 444. Patent Owner's expert, Dr. Quackenbush, published a paper reporting, "[t]he ***CNV segments*** identified by our method range from 120 bp (single exons with higher than average coverage) ***to 240 Mb in size (whole***

chromosomes)." EX1057, 2652. Thus, as Dr. Gabriel testified[7], the ordinary and customary meaning of CNV encompasses aneuploidy. *E.g.*, EX1002, ¶27; EX2021, 59:19-60:4.

Patent Owner's citations to the COSMIC database's "CNV Overview" webpage and Loo are unavailing. POR, 20-21 (citing EX2035, EX2036). While the webpage describes some "specified CNV" at the gene level, Dr. Bustamante conceded that COSMIC does not include all types of actionable CNVs and does not address aneuploidy. EX1050, 36:19-37:5, 37:15-23. Similarly, Loo describes an "***allele-specific*** copy number analysis" (EX2036), which Dr. Bustamante acknowledged was "a specific framing of the allele identification problem in the context of copy number variation." EX1050, 39:14-40:4. Neither the "CNV Overview" webpage nor Loo demonstrates that CNV ***excludes*** aneuploidy.

In view of the overwhelming intrinsic and extrinsic evidence, Patent Owner's complaints about the Board's construction (POR, 18-19) do not withstand

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[7] Patent Owner repeatedly mischaracterizes Dr. Gabriel's testimony to assert that she did not consider the intrinsic record in forming her opinions. *But see* EX1002, ¶¶82, 86, 91, 94-95, 127, 137, 141, 147, 186, 199, 208. Dr. Gabriel correctly interpreted the claim terms in accordance with their ordinary and customary meaning, as understood by a POSA as of the priority date. *Id.*, ¶67.

scrutiny. The Board correctly construed "CNV" according to its ordinary and customary meaning to include aneuploidy.

B. "predefined regions"

As with CNV, the Board correctly rejected Patent Owner's construction of "predefined regions" that excluded entire chromosomes. D.I., 7-8; POR, 11-16. Patent Owner asserts that the term is limited by a single embodiment in the specification that describes "predefined regions" as "at least about" various sizes, including "at least about…100 kb in size." EX1001, 3:61-65, 16:7-12; *see also* POR, 13-14; POPR, 19-20. In so doing, Patent Owner again violates the well-settled principle of claim construction that limitations from single embodiments should not be read into claims. *See* D.I., 7-8; *Phillips*, 415 F.3d at 1323; *Cont'l Circuits*, 915 F.3d at 795-96.

Moreover, as the Board correctly concluded, "while the Specification teaches an embodiment where the predefined regions are limited to a specific range of kilobases, the Specification also uses the term to encompass larger regions that can include chromosomes in a genome." D.I., 7-8 (citing EX1001, 5:22, 10:50, 17:32, 25:2-3). Patent Owner's criticism of the Board's findings are baseless; Patent Owner agrees that a predefined region is found in a genome and does not disagree with the basic principle that a chromosome is part of a genome. POR, 14-15. Thus, consistent with the Board's Institution Decision and the Petition, the

specification supports a broad definition of "predefined regions" that is not "limited to any specific number of base pairs," but rather "encompasses any predefined region of a genome, including an entire chromosome." D.I., 8; Petition, 22, 27, 29; *see Johnson Worldwide Assocs. v. Zebco Corp.*, 175 F.3d 985, 991 (Fed. Cir. 1999). This definition also is consistent with a POSA's understanding of the term. *See* EX1002, ¶¶88, 96; EX2022, 71:8-15, 72:21-24 ("I took 'predefined region' as something pretty straightforward and that someone of skill in the art would understand.").

Patent Owner's remaining contention is that Claim 1, step (e)(i) would be rendered meaningless if "predefined regions" encompasses whole chromosomes, because the claim recites "normalizing a number of reads ***in*** the plurality [of] predefined regions to each other." POR, 11-13. Neither Patent Owner nor its experts provide a single citation to support this assertion. Moreover, this argument is based on two incorrect premises. First, Patent Owner contends that Claim 1 "requires" performing step (e)(i) in a single predefined region in order to determine CNV. *See* POR, 11-13. But as Dr. Bustamante conceded, the use of "and/or" in step (e) demonstrates that the performance of step (e)(i) is ***not*** required.[8] EX1050,

[8] At first, Dr. Bustamante first adamantly stated that "to detect the copy number variation, the first part of the E(i) has to occur. You need to have that

167:14-24. *See also Ex parte Gross*, Appl'n No. 11/565,411, Appeal No. 2011-004811, at 4 (PTAB Jan. 3, 2014) ("We agree with Appellant that 'and/or' covers embodiments having element A alone, element B alone, or elements A and B taken together."). Second, Patent Owner asserts that Claim 1 requires normalizing reads ***in each of*** the predefined regions to each other (i.e., on the same chromosome). *See* POR, 11-13. But the claim language only recites normalizing reads "in ***the plurality of predefined regions***" to each other (i.e., a set of chromosomes). EX1001. Dr. Bustamante also admitted that Claim 1 does not require normalizing reads to determine CNV "in" a single region, but rather "[s]o that you can determine copy number variation ***among*** the plurality of predefined regions." EX1050, 168:3-6. *See also id.*, 28:2-6.

For these reasons, the Board correctly found that "predefined regions" does not exclude entire chromosomes. D.I., 7-8.

C. "unique sequence reads"

The Board mistakenly accepted Patent Owner's out-of-context arguments and citations as supporting a preliminary construction of "unique sequence reads" as "a sequence read that contains unique sequences, such that the sequences can be

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normalization step; okay. That's quite clear." EX1050, 34:21-25. He was then forced to concede the correct meaning of "and/or." *Id.*, 167:14-24.

identified ***without the need for a barcode***." D.I., 12. In so doing, the Board interpreted "unique sequence reads" as "the opposite of" reads that include barcodes. *Id.*, 31-32. Notably, neither party advocated for this construction. Moreover, as Patent Owner's expert agrees, this construction is inconsistent with the '743 patent's claims and specification.

First, nothing in the claims suggests that "unique sequence reads" are defined by whether they include barcodes, and certainly not that they ***exclude*** reads having barcodes. "Barcode" does not appear in the independent claims, but dependent claims 5-7, 9, 15-17, and 19 all expressly recite "attaching one or more barcodes," thus making clear that barcodes are intended to be used in the claimed methods. EX1001. Dr. Bustamante further agreed that the '743 patent discloses determining "unique sequence reads" by "molecular barcoding," including "how you can assign barcodes." EX1050, 137:23-138:5. Thus, a construction of "unique sequence reads" that excludes barcodes is counter to both the claim language and the purported purpose of the '743 patent. *See Trs. of Columbia Univ. in City of N.Y. v. Symantec Corp.*, 811 F.3d 1359, 1370 (Fed. Cir. 2016).

Indeed, the claims only draw a distinction between "mapped reads" and "unique sequence reads." *E.g.*, Claim 1 ("quantifying mapped reads or unique sequence reads…"); EX1050, 129:13-17 (interpreting Claim 10's recitation of "the sequence reads" as "***the mapped*** sequencing reads," which is different from

"unique sequence reads"). As explained in the Petition, sequence reads can be mapped to more than one location on the genome. Petition, 9-10. Identifying reads that can only be mapped to one location allows for the identification of "unique" sequences. *Id.*, 24. For example, Claim 10 requires "***mapping*** sequence reads derived from the sequencing," and then "determining ***unique sequence reads*** **corresponding to the extracellular polynucleotides from among the [mapped] sequence reads**." EX1001, 63:35-39. *See* EX1050, 134:8-16, 136:15-137:8. Thus, as explained in the Petition, a POSA would have understood that "unique sequence reads," as recited in the claims, simply are "sequence reads that can be mapped to just one location," and thus can be determined to correspond to a particular extracellular polynucleotide. Petition, 10, 24. *See also Fin Control Sys. Pty., Ltd. v. OAM, Inc.*, 265 F.3d 1311, 1318 (Fed. Cir. 2001) (citing *Phonometrics, Inc. v. N. Telecom Inc.*, 133 F.3d 1459, 1465 (Fed. Cir. 1998)).

The portions of the specification cited by Patent Owner, on which the Board relied, do not suggest otherwise. In its Preliminary Response, Patent Owner conflated the specification's discussion of "unique sequence ***data***" and the claim term, "unique sequence ***reads***." POPR, 23-24 (citing EX1001, 37:37-44). The Board then relied on this out-of-context discussion in construing "unique sequence reads." D.I., 11-12 (citing EX1001, 37:37-53). The specification, however, simply describes how "unique sequence ***data***" of sequence reads may be used with other

read aspects—as well as barcodes—for "identification of fragments" from a single "parent strand." EX1001, 37:20-63. The cited passages do not discuss, or draw any distinction between, "mapped reads" and "unique sequence reads," as recited in the claims. Instead, as Dr. Bustamante acknowledged, they actually provide ways of "determin[ing] unique sequence reads corresponding to the extracellular polynucleotides" from among the other mapped reads, including through the use of barcodes. *See* EX1050, 136:15-138:5; EX1001, Claim 10. As such, the Board's preliminary construction of "unique sequence reads" should be set aside.

III. GROUND 1: CHIU

A. Claim 1

As an initial matter, Patent Owner does not dispute, and therefore concedes, that Chiu discloses Claim 1(a), (b), (c). *See* POR, 22-28; Petition, 23-25.

Based wholly on its incorrect constructions of "CNV" and "predefined regions," Patent Owner asserts that Chiu does not perform the preamble and step (d) of Claim 1. POR, 22-24, 26-28.[9] Under the correct customary and ordinary

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[9] Patent Owner's cites to Chiu do not demonstrate that CNV fails to encompass aneuploidy. *See* POR, 27. Rather, Chiu merely describes how its method could be readily adjusted to determine ***all*** types of CNV, including "aberrations involving regions less than a whole chromosome." EX1022, 20462.

meaning of these terms, however, Patent Owner does not dispute that Chiu discloses a method for detecting a CNV, i.e., fetal aneuploidy. *See supra* Section II; Petition, 16-31; D.I., 18.

Patent Owner's remaining argument—whether Chiu performs normalization step (e)(i)—is disposed of by a straightforward matter of law. POR, 24-26. Specifically, the use of "and/or" means that an anticipating reference need ***not*** perform ***both*** steps (e)(i) and (e)(ii) in order to "determin[e] copy number variation in one or more of the plurality of predefined regions[.]"[10] EX1001, 62:56-65. *See Brown v. 3M*, 265 F.3d 1349, 1351 (Fed. Cir. 2001) ("When a claim covers several structures or compositions, either generically or as alternatives, the claim is deemed anticipated if any of the structures or compositions within the scope of the claim is known in the prior art."). Thus, Patent Owner's assertion that "Chiu fails to disclose at least step 'e)i)' of claim 1, and thus cannot anticipate claim 1 or any of the challenged dependent claims" (POR, 26) fails as a matter of law.

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[10] Patent Owner incorrectly asserts that the Petition's exemplary claim chart, which merely summarizes how Chiu discloses both steps of Claim 1(e), "mak[es] the processing step 'e)ii)' ***mandatory*** as applied to Chiu." POR, 25. Dr. Bustamante conceded neither step is mandatory. EX1050, 167:14-24.

In any event, the Petition demonstrates that Chiu discloses Claim 1(e) because Chiu determines CNV[11] by performing both normalizing and processing. Petition, 28-30. Patent Owner acknowledges that the Petition establishes that "the processing step of claim 1 [is] allegedly met by a Z–score analysis in the legend to Chiu Fig. 1." POR, 25. To escape this disclosure, Patent Owner conflates the "processing" step (which includes comparing "a number of reads in the plurality of predefined regions" with "numbers obtained from a control sample") with its misreading of the "normalizing" step and argues that, as a result, "meaningful information about aneuploidy is not obtainable." *Id.*, 25-26. As explained in the Petition, however, Chiu discloses comparing "the z-score of % chr21" of a test

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[11] Patent Owner's assertion that Chiu does not determine CNV "***in***" a predefined region but rather "***of***" a predefined region ignores the "one ***or more***" language of the claim. POR, 24; *see supra* pp. 10-12. Claim 1(e) requires determining CNV "in one ***or*** more of the plurality of predefined regions" —i.e., in the set of multiple predefined regions. *See* EX1050, 168:3-6 (Claim 1 does not require determining CNV "in" a single region, but rather "[s]o that you can determine copy number variation ***among*** the plurality of predefined regions."). There can be no dispute that Chiu discloses determining aneuploidy among a set of chromosomes. *See* Petition, 26-30.

sample to a control sample. Petition, 28-29. Thus, Patent Owner concedes that Chiu performs at least step (e)(ii). *See* POR, 24-26.

Moreover, Chiu also discloses normalizing step (e)(i). Normalization was "a routine aspect[] of rare mutation and copy number variation analysis as of September 4, 2012." Petition, 11. Patent Owner does not dispute that "normalization" is a broad term, which the '743 patent does not define, that can refer generally to "methods of adjusting data that allow for comparison of data from different sources." Petition, 27; POR, 12-13, 23-26. Indeed, Dr. Bustamante repeatedly equated "normalization" and "comparison." EX1050, 52:14-25, 53:11-18 ("I would say that in -- in the patent, what we're doing is normalizing the reads in the –[ ] plurality of the predefined regions to each other. And in that process of comparison, one is doing the [ ] normalization."), 151:11-19 (agreeing that a "comparison" of tagged nucleotides is "normalization"). *See also* Motion to Amend (Paper 26, "MTA"), 9 (citing EX2028, ¶221 as support for the "normalizing" step, which includes a comparison of "the amount of tagged parent molecules" mapping to a first and second "location or mappable position"). As discussed in the Petition, Chiu performs normalizing by adjusting data so that reads "for each chromosome can be compared to one another and to the total number of sequence reads." Petition, 27. Accordingly, Chiu anticipates Claim 1.

B. Claims 2-3 and 22-26

Patent Owner offers no specific arguments regarding Claims 2-3 and therefore concedes that Chiu discloses these claims. *See* POR, 22-28.

For Claims 22-26, Patent Owner likewise makes little effort to dispute Chiu's teachings. *See id.* These claims require only that the method of Claim 1 "comprises"[12] (1) step (e)(i) (Claims 23 and 24), (2) step (e)(ii) (Claims 25 and 26), or (3) both steps of claim 1(e) (Claim 22). EX1001, 64:33-47. For the reasons discussed above and in the Petition, Dr. Gabriel's declaration, and the Institution Decision, Chiu anticipates Claims 1-3 and 22-26.

IV. GROUND 2: KINDE 2011 AND KINDE 2012

A. Claim 1

Patent Owner's primary response to Ground 2 also is that (1) Kinde 2012 does not perform the normalization step (e)(i) of Claim 1, and (2) Kinde 2012 does not detect CNV. POR, 29-39. As explained below and in the Petition, these arguments fail. Kinde 2011 and Kinde 2012 thus render Claims 1-9 and 20-26 unpatentable as obvious under Ground 2. *See* Petition, 31-52.

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[12] Claims 22-26's use of "comprises" does not exclude performance of step (e)(ii). *See Genentech, Inc. v. Chiron Corp.*, 112 F.3d 495, 501 (Fed. Cir. 1997); *Solvay S.A. v. Honeywell Int'l Inc.*, 742 F.3d 998, 1005 (Fed. Cir. 2014).

1. Kinde 2012 Discloses Claim 1(c), (d), and (e)

The Board correctly found that Kinde 2012's normalization procedure (EX1041, 4) discloses both mapping (Claim 1(d)) and normalizing (Claim 1(e)(i)). *See* D.I., 26; Petition, 38-39. Patent Owner now asserts that Kinde 2012 does not disclose step (e)(i) because it "normalizes reads among different samples" instead of "in a plurality of predefined regions to each other."[13] POR, 30. Patent Owner is incorrect. First, Dr. Bustamante testified that step (e)(i) can be performed through a comparison of tag counts—precisely what Kinde 2012 teaches. *E.g.*, EX1050, 142:13-144:7, 148:7-13, 149:1-5, 151:11-19.

Second, Dr. Bustamante repeatedly equated "comparing" with "normalizing," thus conceding Petitioner's point. *E.g.*, EX1050, 52:14-25, 53:11-18, 151:11-19. According to Dr. Bustamante, Kinde 2012 teaches a process of "column normalization" that involves "sorting the row in each table based on tag count," "either low to high or high to low." *Id.*, 123:9-125:12. *See* D.I., 26; EX1041, 4. Thus, under Dr. Bustamante's view, Kinde 2012 teaches at least

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[13] At most, the passage Patent Owner cites merely shows that Kinde 2012 performs "normalizing" in order to allow for a comparison among different samples, not that Kinde 2012 is limited to normalization across different samples. *See* EX1041, 6; Petition, 27.

comparing the tag counts within each sample (in order to sort them), i.e., "normalizing a number of reads in the plurality of defined regions to each other." EX1050, 123:9-125:12; MTA, 9; EX2024, ¶42; EX2028, ¶221.

Furthermore, Dr. Bustamante agrees that Kinde 2012 discloses Claim 1(e) by teaching at least the processing step (e)(ii). *See* EX1050, 116:15-17 ("Rather, what Kinde has done is far more similar to what…the patent calls the processing step."), 117:19-118:2; Petition, 38-41; D.I. 25-26. *See supra* pp. 19-20.

Patent Owner's remaining arguments distort the claim language and Petitioner's arguments. Patent Owner contends that by identifying "the human genome (hg19)" as the claimed "reference sequence," Petitioner must demonstrate that Kinde 2012 maps reads to ***every single base*** of hg19 (rather than certain chromosomal positions) in order to render this limitation obvious. POR, 36-38. This nonsensical interpretation is contrary to the specification, which expressly contemplates that "a reference sequence" can be "the human genome," and that reads can be mapped to particular locations or positions in said reference sequence. EX1001, 35:14-22.[14] As the Board correctly found, Kinde 2012 teaches mapping

[14] In contrast, the claims in *Engels Indus. v. Lockformer Co.*, 96 F.3d 1398 (Fed. Cir. 1996), included mutually exclusive language, and thus the two portions of an apparatus could not be the same thing. POR, 38. Similarly, in *Hopkins Mfg. Corp.*

reads to the human genome (hg19) as well as further analysis, including quantifying mapped reads in a plurality of predefined regions of the reference sequence, i.e., specific chromosomal positions. *See* D.I., 25-26; Petition, 37-39.

2. Kinde 2011 and Kinde 2012 Detect CNV

As discussed above, Patent Owner's repeated reliance on its improper construction of CNV fails. *See supra* Section II.A; POR 33-35; Petition, 18-19, 34. There is no dispute that Kinde 2012 teaches a method of detecting fetal aneuploidy (i.e., a CNV).

B. The Petition Establishes Motivation to Combine and Reasonable Expectation of Success

The Petition provides detailed and reasoned evidence of the motivation to combine Kinde 2011 and Kinde 2012 with a reasonable expectation of success, including (1) the references share authors, library preparation, and sequencing techniques, (2) Kinde 2011 discloses that a potential application of its method is prenatal diagnosis using fetal DNA from maternal plasma (as reflected in Kinde 2012), and (3) Kinde 2012 expressly incorporates Kinde 2011's teachings and

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v. Cequent Performance Prod., Inc., IPR2015-00613, Paper 19 (PTAB Aug. 7, 2015), the Board noted that while a single structure may satisfy two claimed limitations, the specification at issue ***required*** that the two claimed signals be separate and distinct. *Id.*, 11-12. The '743 patent has no such requirement.

highlights their advantages. *See* Petition, 31-34. The Petition further explains that a POSA would have been motivated to apply the techniques of Kinde 2012 and Kinde 2011 to detect "relatively rare mutations" (which may include CNVs), because a disclosed advantage of Kinde 2011's method is the ability to determine the "number of templates," which is "a key aspect of copy number analysis." Petition, 33; EX1023, 9350. Dr. Gabriel's testimony further supports that a POSA would have recognized these advantages and reasonably expected success using these techniques. *See* EX1002, ¶¶100-106; EX2021, 185:11-186:16. Moreover, the Board already acknowledged the "reasonable inferences an ordinary artisan would make from [the Kinde 2012 and Kinde 2011] references." D.I., 25. *See id.*, 23-25; *Med. Instrumentation & Diagnostics Corp. v. Elekta AB*, 344 F.3d 1205, 1221-22 (Fed. Cir. 2003); *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 418 (2007). In view of these teachings, Patent Owner's arguments should be rejected.

C. Dependent Claims 3-7 and 9 Are Also Unpatentable

Patent Owner's arguments regarding Claims 3-7 and 9 are based entirely on Dr. Quackenbush's unsubstantiated opinions, none of which held up during deposition. POR, 42-45. First, for Claim 3, Dr. Quackenbush admitted that Kinde 2011 discloses generating copies of DNA fragments through amplification prior to sequencing. EX1051, 26:6-16. While Dr. Quackenbush criticizes Kinde 2011's methods as purportedly being "inapplicable" to "extracellular polynucleotides"

(i.e., cell-free DNA) (EX2026, ¶28), he ignores that (1) it is the combination of Kinde 2011 ***and*** 2012 that renders Claim 1 (from which Claim 3 depends) obvious, (2) nothing in Kinde 2011 indicates that its methods are "inapplicable" to cell-free DNA (EX1051, 29:16-21; *see also* EX1023, 9530; EX1050, 132:23-25), and (3) Kinde 2012 expressly analyzes extracellular polynucleotides. *See* Petition, 34-35, 42.

Second, Dr. Quackenbush asserted that Kinde 2012 detects aneuploidy and not a "rare mutation" according to Claim 4, but then admitted that he did not consider if "rare mutation" could include CNV or aneuploidy in the context of the '743 patent. EX2026, ¶32; EX1051, 48:13-49:13. Dr. Quackenbush further does not dispute that Kinde 2011 discloses calculating the percent of sequences having a rare mutation, as stated in the Petition. *See* Petition, 1043; EX2026, ¶¶31-32.

Third, Dr. Quackenbush's opinions regarding Claims 5-7 and 9 are all based on purported distinctions between allegedly "low-abundance, low concentration" cell-free DNA and "abundant" cellular DNA. POR, 43-44; EX2026, ¶33. However, Dr. Quackenbush ultimately admitted that nothing in these claims actually requires any particular quantities of polynucleotides. EX1051, 84:5-14, 85:17-99:3. Moreover, although Kinde 2012 does disclose cellular DNA, it also expressly analyzes cell-free DNA, which Dr. Quackenbush curiously ignores. *See* Petition, 43. *Compare* EX1049, Abstract *with* EX1051, 31:2-34:4, 35:17-37:16.

Fourth, Dr. Quackenbush's arguments regarding Claims 5-7 and 9 are simply not credible. In particular, Dr. Quackenbush argues that "Kinde 2011 tags amplification progeny—not polynucleotides from a subject which is recited in the claim." EX2026, ¶34; POR 44. But Patent Owner adamantly argued in the District Court litigation—and convinced the Magistrate Judge during claim construction—that "extracellular polynucleotides" include amplicons. *See* EX1058, 2-3; EX1059, 12-13 ("The crux of the parties' dispute here is whether this term…***encompasses the sequencing of amplified copies of such polynucleotides (as Guardant asserts***)."). Because Claims 5-7 and 9 recite attaching barcodes "to the extracellular polynucleotides," and Patent Owner itself has maintained that such polynucleotides encompass amplicons, Dr. Quackenbush's assertion that Kinde 2011 does not teach this limitation is meritless. *See also* Petition, 44-47.

Finally, Dr. Quackenbush's remaining arguments that Kinde 2012 does not produce "sheared ends required by the endogenous UID embodiment of Kinde 2011" is premised on an incorrect assumption that Kinde 2012 must produce sheared ends to render the claims obvious. EX2026, ¶36; POR, 45. Dr. Quackenbush ignores that Kinde 2011 discloses using exogenous sequences in the Inverse PCR experiment, and that it would have been obvious to substitute the single primer in Kinde 2012 with the exogenous sequence primers of Kinde 2011

to (a) maintain diversity at the end to each fragment and (b) add non-unique identifier sequences. *See* Petition, 46-50. Accordingly, Kinde 2011 and Kinde 2012 also render Claims 3-7 and 9 obvious.

V. GROUND 3: KINDE 2011 AND FORSHEW

Patent Owner's cursory response to Ground 3 merely cites the Board's Institution Decision, which, as discussed above, relies on an incorrect construction of "unique sequence reads." *See* Section II.C. Notably, Patent Owner's response does not adopt this construction. POR, 46-47. Instead, Patent Owner asserts that Forshew does not disclose "unique sequence reads" as recited in Claim 10(f), without addressing the Board's construction at all.[15] This argument fails.

First, the challenged claims are rendered obvious by Kinde 2011 ***and*** Forshew. *See* Petition, 53-69. With respect to Claim 10(d), Kinde 2011 expressly teaches use of unique identifiers (UIDs) to determine unique sequence reads that correspond to the extracellular polynucleotides from among the mapped sequence reads. *See* Petition, 58-59. For Claim 10(f), Forshew teaches a method that includes identifying subsets of mapped reads that include a variant for each of multiple mappable base positions. *Id.*, 60-63. Forshew further calculates an allele

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[15] Patent Owner does not dispute that Kinde 2011 teaches "unique sequence reads." *See* POR, 32-33, 46-51.

frequency (i.e., a ratio of mapped reads containing variants to a total number of reads for each of numerous specific genomic locations). *Id.*, 60. As Petitioner explained, a POSA would have been motivated to combine Kinde 2011 and Forshew for many reasons, including that Kinde 2011 provides an improved method for achieving the precise goal identified by Forshew, i.e., reducing false positives. *Id.*, 53-55. Thus, as described in the Petition, Claims 10-13, 15-19 and 21 are obvious over Kinde 2011 and Forshew. *See* Petition, 53-69.

VI. GROUND 4: KINDE 2011, KINDE 2012, AND FORSHEW

Claim 14 depends from Claim 10 and is also obvious over the teachings of Kinde 2011, Kinde 2012 and Forshew. *See supra* Section V; Petition, 69-70.

VII. NO OBJECTIVE INDICIA OF NON-OBVIOUSNESS

Patent Owner has not raised, and thus has waived, any objective indicia of non-obviousness. *See* Petition, 71; EX1050, 18:2-19; EX1051, 14:11-15; EX1002, ¶¶232-40.

VIII. CONCLUSION

For these reasons and as stated in the Petition, Claims 1-26 should be cancelled.

Respectfully submitted,

Date: March 3, 2020

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CERTIFICATE OF COMPLIANCE

I hereby certify that the word count for the foregoing Petition totals **5600** words, excluding the parts which are exempted by 37 C.F.R. § 42.24(a)(1).

CERTIFICATE OF SERVICE

The undersigned hereby certifies that the foregoing **PETITIONER'S REPLY TO PATENT OWNER RESPONSE** was served electronically in its entirety on March 3, 2020 via electronic mail to the following attorneys of record:

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

FOUNDATION MEDICINE, INC.,
Petitioner,

v.

GUARDANT HEALTH, INC.,
Patent Owner.

Case No. IPR2019-00652
Patent No. 9,834,822

PATENT OWNER RESPONSE

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I. STATEMENT OF PRECISE RELIEF REQUESTED

Foundation Medicine, Inc. ("Petitioner") filed a petition for inter partes review of claims 1-13 and 17-20 of U.S. Patent No. 9,834,822 ("the '822 patent," EX1001). The Board issued its decision instituting trial (Paper 12) on all grounds set forth in the petition. Guardant Health, Inc. ("Patent Owner") hereby requests that the Board now issue a final written decision confirming that claims 1-13 and 17-20 are not unpatentable.

II. INTRODUCTION

The '822 patent claims novel methods for detecting rare mutation in cell-free DNA (cfDNA) samples. Detecting rare mutation in cfDNA presents a considerable challenge — a challenge derived from both the physical properties and minute quantities of cfDNA found in clinical specimens. The '822 patent covers Patent Owner's commercial product, the Guardant360 genomic test. Guardant360 provides highly sensitive detection of genetic aberrations using the non-unique barcoding method of the '822 patent. The Guardant360 test has been a commercial success and received tremendous recognition in the field, including being granted Breakthrough Device designation by the FDA.

The petition materials present a single ground of challenge based on references to Schmitt et al. (EX1011 and 1047). According to Petitioner, Schmitt discloses a barcode-based error correction method that satisfies all aspects of the

challenged claims, but for its application to cellular DNA rather than cfDNA as claimed. Petitioner avers that one would have been motivated, with the requisite expectation of success, to apply Schmitt's method to cfDNA as such a sample was known at the time, as evidenced by Fan (EX1048) and Forshew (EX1004). The petition challenges fail for numerous reasons.

First, the petition materials fail to establish that the cited reference teach all aspects of the challenged claims. This is particularly apparent for "non-uniquely tagged parent polynucleotides" as required by claim 1.

For this aspect, the Petitioner asserted, and the Board agreed in its Institution Decision, that the '822 patent provides an express definition of "non-uniquely tagged." Specifically, both Petitioner and the Board indicate that the '822 patent expressly defines the term in explaining that parent polynucleotides "can be non-uniquely tagged, that is, the number of different identifiers can be at least 2 and fewer than the number of polynucleotides ***that map to the mappable base position***." Pet. 21 (citing EX1001, 41:42-46); Paper 12 at 9-10.

Yet, the petition materials (and the Institution Decision), instead of applying what is identified as an express definition, wholly ignore the phrase "***that map to the mappable base position***" in order to assert that "non-uniquely tagged" is found in the cited art. As a result, the petition case (and the Institution Decision) erroneously focus on an entire population of polynucleotides in a sample, which is

far more expansive than "polynucleotides that map to the mappable base position." This disconnect between the recognized claim scope and the prior art application infects the entire analysis in the petition. More fundamentally, this illustrates that the petition materials fail to meet basic requirements of a *prima facie* showing, let alone meet Petitioner's burden of demonstrating the challenged claims unpatentable. The petition challenge can be rejected for this reason alone.

Should further analysis be required, it is clear that Schmitt fails to meet this aspect of the challenged claims. The method of Schmitt expressly requires flooding a sample with "SMI tags" or "n-mers" to ensure that every DNA fragment in the sample is uniquely labeled—an aspect critical to Schmitt's method. There simply is no disclosure in Schmitt that would instruct non-unique labeling, and Petitioner's efforts to depart from the content of the reference and contrive unreasonable hypotheticals cannot change this fact.

Beyond the lack of "non-uniquely tagged," the cited art fails to teach "converting the population of cfDNA molecules into…tagged parent polynucleotides," as required by the challenged claims. Not only does Schmitt not apply its methods to cfDNA, it critically employs a shearing technique that even Petitioner's expert, Dr. Gabriel, concedes is incompatible with cfDNA samples. The secondary references do not cure this deficiency, as Forshew only tags PCR

amplicons (not "parent polynucleotides") and Fan does not employ tagging at all. This represents another reason why the petition challenges fail.

Finally, should the Board reach the issue, the challenges also fail to establish that one would have been motivated to apply the method of Schmitt to cfDNA as asserted with any reasonable expectation of success. Petitioner offers nothing beyond unsupported assertions and false characterizations of the prior art. As explained in detail by Patent Owner's expert witnesses, Dr. Quackenbush and Dr. Shendure, a person of ordinary skill in the art ("POSA") would have recognized Schmitt as being poorly suited for cfDNA analysis. Indeed, the testimony of all experts (including Dr. Gabriel), relevant literature, and even the content of Schmitt itself indicates there are critical aspects of the Schmitt method (*e.g.*, large sample requirements, optimal fragment size, random shearing, random degenerate tags, numerous processing steps) that would have been recognized as incompatible with cfDNA sample analysis.

Accordingly, the petition challenges should be rejected and the '822 patent claims be found not-unpatentable.

III. BACKGROUND

A. Cellular DNA and cell-free DNA

While cfDNA was known to exist, it was also well-known that cfDNA presents unique technical difficulties compared to analysis of cellular DNA. Unlike

cellular DNA, cfDNA is present in such low concentrations and purity that detection and analysis was difficult with prior techniques. *E.g.*, EX1048 at 16270 (disclosing cfDNA samples containing between 1 and 8 ng); EX1001, 33:12-17 (teaching that prior art library preparation methods were low efficiency and required "greater than 1 microgram of DNA as input material."). Additionally, the highly fragmented nature compounds the difficulty of working with the minute amounts of cfDNA in samples. *See also* EX2025, ¶¶26, 27.

Even Petitioner's own witness, Dr. Gabriel, has described cfDNA analysis in her publications as presenting unique technical challenges:

> Furthermore, the sensitivity to detect variants from low purity samples will be limited by the total yield or genome equivalents of cfDNA that are available for sequencing. Thus, the accurate profiling of tumor DNA or RNA in a sample that contains non-tumor DNA or RNA is ***challenging and requires specialized methods***…

EX2003 at 3

Absent a showing to the contrary, those of skill in the art at the time were skeptical that a method designed for cellular DNA would be suitable for analysis of cfDNA. EX2002 at 102 ("Prospects of success are limited since the [Schmitt] method is relatively inefficient when limited amounts of input DNA—as it is most likely the case for cfDNA—are used"); *see also* EX2025, ¶28.

B. Next Generation Sequencing and Error Correction Techniques

Next Generation Sequencing ("NGS") has been used in a wide array of different methods. Most NGS-based studies and diagnostic assays do not use an error correction method. This is because the error rate associated with NGS is sufficiently low such that it does not significantly affect interpretation of the resulting data. *See, e.g.*, Fan (EX1004); EX2025, ¶¶29-33.

Error correction techniques add considerable cost and complexity. For example, error correction increases the amount of sequencing capacity needed to analyze a given target. Error correction significantly increases sequencing requirements and costs, as it involves sequencing of multiple amplicons that derive from each strand of the original sample polynucleotide. EX1011, 16:20-25. Moreover, error correction requires additional bioinformatic steps (*e.g.*, grouping, generating a consensus sequence) which adds to the cost and complexity. *E.g.*, EX1011, 4:4-29; *see also* EX2025, ¶¶34-36.

Furthermore, as explained by Dr. Quackenbush, barcode techniques can skew test results in indiscernible ways. For reasons not completely understood, disparities in performance are often observed among different barcodes. This is particularly problematic with degenerate barcodes. *See also* EX2025, ¶37.

Finally, error correction schemes introduce inefficiency and often lose a considerable amount of sample. EX1057 at 5. Sample loss may be tolerable when

working with samples containing large amounts of DNA (*e.g.*, microgram quantities), but is not acceptable when working with samples that contain only a minute amount of DNA — such as in the case of cfDNA. EX1001, 60:50-61; *see also* EX2025, ¶¶38-39.

IV. CITED REFERENCES

A. Fan (EX1048)

Fan discloses detection of fetal aneuploidy in maternal plasma samples using shotgun sequencing. EX1048 at 16266. Fan does not disclose detecting sequence variants in maternal plasma samples. Rather Fan discloses determining fetal aneuploidy by detecting an excess of NGS reads associated with a given chromosome. EX1048 at 16267-68; *see also* EX2025, ¶40.

Fan does not disclose tagging sample DNA nor does it disclose a method for correcting sequencing errors. EX1048 at 16270, 16271; *see also* EX2025, ¶41.

B. Forshew (EX1004)

Forshew discloses accurate detection of mutant alleles at frequencies as low as 2%. EX1004 at abstract, Table 1, 3 ("Quantitative limitations of mutation detection"). Forshew uses deep sequencing and replicate analysis to avoid false positives resulting from sequencing errors. EX1004 at 9 ("Duplicate sequencing of each sample is used to avoid false positives stemming from PCR errors."), 10 ("Higher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols."), 19 ("A point

mutation was called in a sample if it ranked among the 6 loci with highest non-reference frequency in both duplicates, and was represented by at least 10 reads in each."). *See also* EX2025, ¶¶42-43.

To the extent Forshew teaches tagging, it is the tagging of PCR amplicons for the purpose of multiplex analysis. EX1004 at 2 ("Finally, sequencing adaptors and sample-specific barcodes were attached to the harvested amplicons in a further PCR."), 9 ("Sample barcodes and high throughput PCR reduce the per sample costs to a range where this may be widely applicable."). *See also* EX2025, ¶44.

C. Schmitt (EX1011 and EX1047)

The Schmitt patent (EX1011) and Schmitt 2012 (EX1047) (collectively, "Schmitt")[1] disclose "Duplex Sequencing" which purports to reduce error through analysis of both strands of a DNA duplex. EX1047 at Abstract. *See also* EX2025, ¶45.

The method of Schmitt requires flooding the sample with "SMI tags" or "n-mers" to ensure that every DNA fragment in the sample is uniquely labeled. *E.g.*,

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[1] The petition asserts that the Schmitt (EX1011) and Schmitt 2012 (EX1047) teach "the same DCS method." Pet. 28. Petitioner relies nearly exclusively on the Schmitt patent, however, since both references disclose the same DCS method, Patent Owner's responsive arguments apply equally to both.

EX1011, 3:44-53, 6:59-63 ("The first and/or second degenerate or semi-degenerate nucleotide ***n-mer sequences may be any suitable length to produce a sufficiently large number of unique tags*** to label a set of sheared DNA fragments from a segment of DNA.") (emphasis added), 18:36-41; EX1047 at 14509. *See also* EX2025, ¶46.

Uniquely tagging polynucleotide fragments of the sample is critical to Schmitt's method. Following amplification of the SMI tagged fragments, Schmitt discloses grouping PCR progeny into families solely by virtue of the SMI tag. *E.g.*, EX1011, 4:4-9, 18:36-41, 20:50-52. Because the SMI tag provides the sole basis for grouping sequence reads, each SMI tag must be unique. EX1011, 3:47-49, 6:59-63, 18:36-41, 21:59-61. As such, polynucleotides that are not uniquely tagged cannot be properly grouped and analyzed and must be discarded. *See also* EX2025, ¶¶47-50.

V. THE '822 PATENT

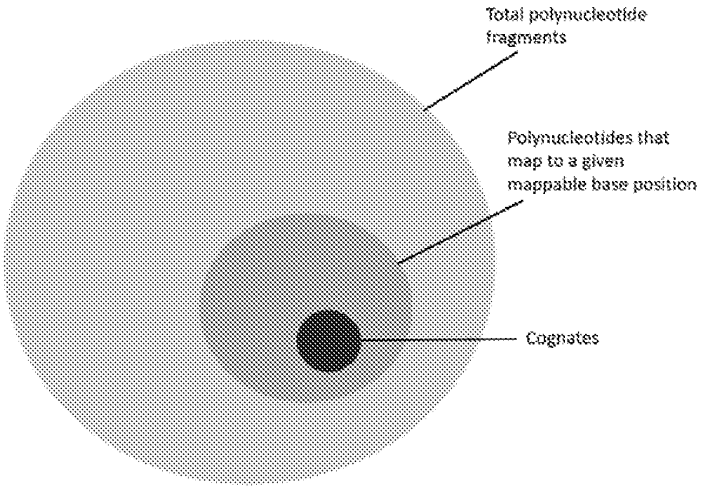
The method of the '822 patent uniquely identifies parent polynucleotide molecules yet does so using only a small number of barcodes. *E.g.*, EX1001, 41:25-28, 41:31-34 ("For example, a sample comprising about 10,000 haploid human genome equivalents of cfDNA can be tagged with about 36 unique identifiers. The unique identifiers can comprise six unique DNA barcodes."). *See also* EX2025, ¶51.

The disclosed method, in part, takes advantage of the inventor's insight that many polynucleotide molecules are distinguishable on the basis of mapping to different positions of a reference sequence. *E.g.*, EX1011, 39:12-18, 40:31-39, 40:61-67 ("This problem [of distinguishing identical molecules] can be diminished by tagging parent molecules with a sufficient number of unique identifiers (e.g., the tag count) such that there is a likelihood that two duplicate molecules, i.e., molecules having the same start and stop positions, bear different unique identifiers so that sequence reads are traceable back to particular parent molecules."). *See also* EX2025, ¶52.

Pursuant to the '822 patent method, only polynucleotide molecules having the same start and stop position relative to the reference sequence ("cognates" or "duplicates") need be distinguished with barcodes. EX1001, 40:4-8, 41:6-13; *see also* EX2025, ¶53. The inventor of the '822 patent determined empirically that the number of cognates in a sample is proportional to the size distribution of the polynucleotide fragments and the number of genomic equivalents—that is, the number of haploid genome copies present in the sample. EX1001, 39:24-29 ("It has been empirically determined that in a sample of about 10,000 haploid genome equivalents of human DNA, there are about 3 duplicate polynucleotides beginning at any given position. Thus, such a collection can contain a diversity of about $6x10^{10}$-$8x10^{10}$ (about 60 billion-80 billion e.g., about 70 billion ($7x10^{10}$))

differently sequenced polynucleotide molecules."); *see also id.*, 40:31-38; *see also* EX2025, ¶54.

The '822 patent distinguishes between (a) total polynucleotide fragments in a sample, (b) polynucleotides that map to a given mappable position in the reference genome, and (c) cognates or duplicates. (*see, e.g.*, illustration). *See also* EX2025, ¶55. For example, cfDNA has a peak size of about 160 nucleotides. EX1001, 40:11-14. A "cfDNA [sample] from a genome of about 3 billion bases (e.g., the haploid human genome) may be comprised of almost 20 million ($2x10^7$) polynucleotide fragments." *Id.*, 40:14-17. Each of the 20 million polynucleotide fragments in a sample comprising one haploid genome equivalent (*i.e.*, 3 billion bases of the human genome), is unique — that is, fragments map to different locations and there are no cognates. However, if the number of haploid genome equivalents is increased (*e.g.*, 10,000 genome equivalents), there is a possibility for cognates in the sample. The '822 patent discloses that when the sample has 10,000 genome equivalents, 3 cognates



are typically found at any given mappable base position. EX1001, 40:18-27; *see also id.*, 40:36-61; EX2025, ¶¶56-57.

According to the '822 patent, the number of tags used in the claimed method depends—not on the total fragments in a sample—but on a subpopulation of fragments that map to a given position in the reference genome and the expected number of cognates. EX1001, 41:6-13. Because only a small number of barcodes are needed using the disclosed non-unique tagging method, these barcodes may be selected and tested in advance. *E.g.*, EX1011, 41:31-36. This provides the additional advantage of using a well-characterized set of barcodes, further enhancing the ability for detecting rare variants in samples comprising cfDNA. EX1001, 60:50-61; *see also* EX2025, ¶¶58-60.

VI. CLAIM CONSTRUCTION

As the Federal Circuit has explained, the ordinary meaning governs interpretation of claim scope, unless the inventor acts as his own lexicographer and assigns a particular meaning to a claim term. *Phillips v. AWH Corp.*, 415 F.3d 1303, 1316 (Fed. Cir. 2005) ("Consistent with that general principle, our cases recognize that the specification may reveal a special definition given to a claim term by the patentee that differs from the meaning it would otherwise possess. In such cases, the inventor's lexicography governs."); *Vitronics Corp. v. Conceptronic*, 90 F.3d 1576, 1582 (Fed. Cir. 1996) (citing *Hoechst Celanese Corp.*

v. BP Chems. Ltd., 78 F.3d 1575, 1578 (Fed. Cir. 1996)); *Cont'l Circuits LLC v. Intel Corp.*, 915 F.3d 788, 796 (Fed. Cir. 2019) ("When the patentee acts as its own lexicographer, that definition governs.").

In this case, the Petitioner asserted, and the Board agreed, that the '822 patent provides an express definition of "non-uniquely tagged." Both the Petitioner and the Board, however, erred in the analysis of the '822 patent claims vis-à-vis the cited art at least because they abandon the very construction identified as an express definition. That is, in analyzing the cited art both the petition materials and the Board's institution decision fail to ever apply the identified express construction. This fundamental disconnect between the recognized claim scope and prior art application infects the entire analysis.

Both Dr. Gabriel and the Board's institution decision state that the '822 patent provides an express definition for the term "non-uniquely tagged." That is, both indicate that the '822 patent expressly defines the term in explaining that parent polynucleotides "can be non-uniquely tagged, that is, the number of different identifiers can be at least 2 and fewer than the number of polynucleotides ***that map to the mappable base position***." EX1002, ¶75 (citing EX1001, 41:42-46); Pet. 21; *see also* EX2025, ¶61.

The Board adopted this as an express definition:

> In the context of "non-unique" identifiers, the '822 patent follows the words "non-uniquely tagged" with "*that is*, the number of different

identifiers can be at least 2 and fewer than the number of polynucleotides that map to the mappable base position."

Paper 12 at 9 (citing EX1001, 41:42-46) (emphasis in original). The Board went on to state that "[w]e read this description in the '822 patent forth an express definition of 'non-uniquely tagged.'" Paper 12 at 10; *see also* EX2025, ¶62.

There is no dispute as to the express definition of "non-uniquely tagged" in the '822 patent. Dr. Quackenbush agrees with Dr. Gabriel and the Board that the above definition for the term "non-uniquely tagged" is appropriate. *See also* EX2025, ¶63. However, Dr. Quackenbush explains that the analysis in the petition materials and the Board's institution decision never apply this interpretation when assessing the cited art.

Express Definition of "Non-Uniquely Tagged" Identified by Petitioner and the Board	Erroneous Application in Reviewing the Cited Art
"the number of different identifiers is at least 2 and fewer than the number of polynucleotides ***that map to the mappable base position***."	"the number of different identifiers is fewer than the number of parent polynucleotides"

Specifically, the petition materials and the Institution Decision, instead of applying what is identified as an express definition, ignore the phrase "***that map to the mappable base position***" in order to conclude that "non-uniquely tagged" is

found in the cited art. *See* Pet. 21 (citing EX1002, ¶75); Paper 12 at 10 ("[W]e further agree with Petitioner that, because the number of different identifiers is fewer than the number of parent polynucleotides, not every parent polynucleotide is tagged with a unique identifier."). Thus, the petition materials and the Institution Decision erroneously focused on an entire population of polynucleotides in a sample, which is far more expansive than a subpopulation of "polynucleotides that map to the mappable base position." *See also* EX2025, ¶64.

"Mappable base position"

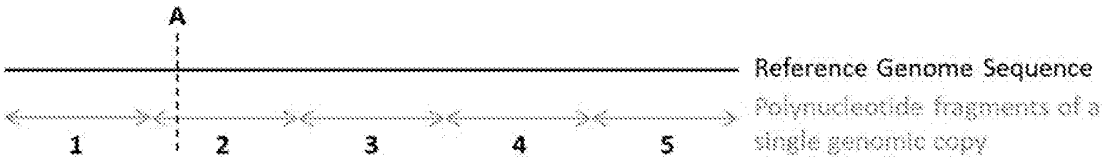
There is no dispute with regard to the meaning of "mappable base position." Dr. Quackenbush agrees with Dr. Gabriel that a POSA would have understood that "mappable base position" is a position in the reference sequence to which polynucleotide molecules can be confidently mapped. EX2027, ¶67; EX2022, 23:22-24:13, 30:4-18, 35:5-7; EX1001, 41:42-46; *see also id.*, 35:1-11; EX2025, ¶65.

According to the express definition of "non-uniquely tagged," the number of different identifiers is determined by the number of polynucleotides that map to the mappable base position.

> A set of polynucleotides in the composition that map to a mappable base position in a genome can be non-uniquely tagged, that is, the number of different identifiers can be at least [] 2 and fewer than the number of polynucleotides ***that map to the mappable base position***.

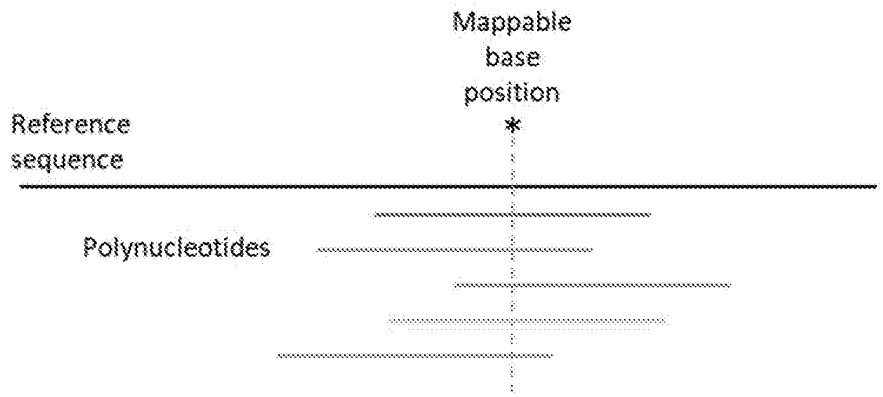
EX1001, 41:42-47. The number of polynucleotides in a sample is different than the number of polynucleotides in a sample that map to the mappable base position. *See e.g.*, EX2022, 56:15-21; *see also* EX2025, ¶¶66-67.

For example, for illustrative purpose, consider a single copy of a genome that is fragmented into five polynucleotides, all of which are confidently mappable to the reference sequence. Such a sample includes, at most, five polynucleotides. Yet, among the total population of parent polynucleotides, there is only one polynucleotide fragment (fragment "2" in this example) that maps to a given mappable base position of the reference sequence (*e.g.*, tumor marker locus "A," below). *See also* EX2025, ¶68.



As another example, consider a sample comprised of five haploid copies of the human nuclear genome that are fragmented into 200 base pair polynucleotides. The entire sample might comprise millions of polynucleotide fragments ((3 billion base pairs x 5 copies)/200). However, at any given mappable base position there can only be 5 polynucleotides (as shown below). The 65,536 possible unique tags from a 4-mer tag sequence might be less than the entire number of polynucleotides in the sample, as asserted by Petitioner, but it is much larger than the five

polynucleotides that map to a given mappable base position. *See, e.g.*, Pet. 44; *see also* EX2025, ¶69.



VII. THE PETITION FAILS

The petition challenges claims 1-13 and 17-20 of the '822 patent as unpatentable over Schmitt in view of Schmitt 2012, further in view of Fan or Forshew. Pet. 30. The petition fails at least because various aspects of the challenged claims are missing from the cited references. Schmitt does not teach "non-uniquely tagged parent polynucleotides" nor do the references teach "converting the population of cfDNA molecules into … tagged parent polynucleotides" as required by claim 1. *See also* EX2025, ¶70.

Furthermore, the petition fails to establish motivation to combine the references as proposed with a reasonable expectation of success. While it is not Patent Owner's burden to prove no motivation and/or expectation of success, that is the only reasonable conclusion in view of the evidence. Even Petitioner's own

expert concedes that a POSA would not have considered Schmitt reasonably applicable to cfDNA. *See also* EX2025, ¶71.

A. Schmitt does not disclose "non-uniquely tagged parent polynucleotides" as required in the challenged claims

Petitioner fails to demonstrate that the cited references teach "non-uniquely tagged parent polynucleotides" as recited in the challenged claims. *See, e.g.*, EX1001, claim 1(b). But the cited DCS method of Schmitt (including the so-called "hybrid" embodiment) critically relies on unique tagging. That is, Schmitt relies on its unique identifiers added to parent fragments in a sample to group together amplification duplicates for sequencing and construction of a consensus sequence for the corresponding parent fragments.

Lacking any disclosure in Schmitt of "non-uniquely tagged parent polynucleotides"—as that aspect of the claims that is undisputedly defined—the petition materials rely on (1) disregarding the express definition of "non-uniquely tagged" and, (2) a scenario constructed from assumptions that find no support in Schmitt.

Specifically, Petitioner's analysis erroneously focuses on an entire population of polynucleotides in a sample, which is far more expansive than "polynucleotides that map to the mappable base position." *See, e.g.*, Section VI. While it is not Patent Owner's burden to rebut a case that Petitioner never made (as is the scenario here), the fact is that Schmitt is silent regarding a sub-population of

fragments in a sample that map to a given mappable base position. As explained by Dr. Quackenbush, the critical absence of such content from Schmitt is unsurprising considering it would be irrelevant and, in fact indeterminable in Schmitt's method. *See also* EX2025, ¶72.

i. *Petitioner never applies the express definition of "non-uniquely tagged" to the prior art*

Petitioner bears the unshifting burden of demonstrating, in the petition, that the challenged claims are unpatentable. *In re Magnum Oil Tools Int'l, Ltd.*, 829 F.3d 1364, 1376 (Fed. Cir. 2016). It is axiomatic that a petitioner's burden of proof has not been satisfied where it identifies an express definition of a critical claim term, yet fails to ever apply that construction in its analysis of the cited art.

The Board's rules identify basic content of a petition critical to establishing a *prima facie* case of unpatentability. This includes, *inter alia*, "[h]ow the challenged claim is to be construed" as well as the following:

> How *the construed claim* is unpatentable under the statutory grounds identified…[and]… The petition must specify where each element of the claim is found in the prior art patents or printed publications relied upon."

37 C.F.R. §42.104(b)(3) & (4); *see also* 35 U.S.C. §312(a)(3).

Here, it is not disputed that the '822 patent expressly defines the term "non-uniquely tagged." *See* Section VI. Petitioner, however, never addressed, let alone

applied, the express definition of "non-uniquely tagged" when addressing its application to the cited prior art.

Rather, Petitioner consistently assumed an interpretation of "non-uniquely tagged" that is different and considerably broader than the express definition. That is, the petition materials erroneously address an entire population of polynucleotides in a sample, rather than "polynucleotides that map to the mappable base position." This error is illustrated throughout the petition materials. *See, e.g.*, Pet. 11-12 ("An alternative approach is to use fewer barcodes than ***original DNA fragments***, such that not every fragment has a unique barcode (i.e., is 'non-uniquely tagged')."), 21 ("The term 'non-uniquely tagged' means that 'the number of different identifiers attached to the polynucleotides is at least 2 and fewer than the number of polynucleotides.'"), 44 ("probability dictates that each tag sequence will be ligated more than once, which means that each tag sequence is non-unique ***relative to a fragmented genome***") (emphases added).

As required by statute, the Board instituted the case as it was presented in the petition. *See e.g.*, Paper 12 at 10 (citing Pet. 11-12, EX1001, ¶¶50-51) ("…because the number of different identifiers is fewer than the number of parent polynucleotides, not every parent polynucleotide is tagged with a unique identifier…"); *SAS Inst., Inc. v. Iancu*, 138 S. Ct. 1348, 1356 (2018).

Because Petitioner never applies the express construction of "non-uniquely tagged," the petition materials fail to meet the basic requirements of a *prima facie* showing, let alone satisfy the burden of demonstrating the challenged claims unpatentable. *See, e.g.*, Trial Practice Guide August 2018 Update at 14 ("Petitioner may not submit new evidence or argument in reply that it could have presented earlier, e.g., to make out a prima facie case of unpatentability."). As such, the petition materials are incurably deficient, and should be rejected on at least this basis.

ii. *Petitioner's contrived scenario does not support its assertion that Schmitt teaches non-uniquely tagged parent polynucleotides*

While Schmitt is clear that uniquely tagging polynucleotides in a sample is critical to the DCS method, it is silent regarding a sub-population of fragments in a sample that map to a given mappable base position. As explained by Dr. Quackenbush, such information unnecessary to Schmitt's method and, in fact, would be indeterminable in the context of Schmitt. *See also* EX2025, ¶72.

Unable to find disclosure of "non-uniquely tagged parent polynucleotides," Petitioner constructs a scenario based on a series of unexplained assumptions:

> Schmitt's "hybrid" approach for tagging DNA fragments results in non-uniquely tagged parent polynucleotides. In this embodiment, if the tag sequence is a 4-mer, for example, then the total number of possible unique tag sequences that may be attached on both ends is only 65,536. A human genome has about 3 billion base pairs, and Schmitt teaches selection of fragments in the optimal range of 200-

> 500 base pairs, which would result in millions of fragments per genome. Given that there are no more than 65,536 possible tag sequences in Schmitt's "hybrid" approach, probability dictates that each tag sequence will be ligated more than once, which means that each tag sequence is non-unique relative to a fragmented genome or to naturally fragmented cfDNA, which has an average size of about 140 to 170 base pairs.

EX1002, ¶127 (internal citations and footnote omitted); Pet. 44 (similar). This scenario does not support Petitioner's assertion that Schmitt teaches non-uniquely tagging. *See also* EX2025, ¶73.

As explained above, the petition identifies an express definition of "non-uniquely tagged," but ignores the meaning of that term in addressing Schmitt. Specifically, Petitioner's analysis erroneously focuses on "millions of fragments per genome" but overlooks the fact that those fragments do not map to the same mappable base position. *See* Section VI. This was confirmed by Dr. Gabriel during cross-examination:

> Q. If one creates a library of fragments from a single copy of a genome, would all of the fragments map to the same given mappable base position in the referenced genome?
>
> MS. SHONEWALD: Objection.
>
> A. No.
>
> EX2022, 56:15-21.

Petitioner's contrived scenario illustrates the disconnect between the undisputed express construction, and Petitioner's arguments ignoring that construction.

For instance, the number of polynucleotides that map to a mappable base position is different than the total number of polynucleotides in a sample, and would require multiple copies of similarly mappable fragments in the sample. *See* Section VI. But Petitioner's scenario comprises fragments derived from one haploid human genome (*i.e.*, 3 billion base pairs). Under that scenario, there could be no more than one polynucleotide at any given mappable base position (and certainly less than 65,536). *See* Section VI. With only one polynucleotide at a given mappable base position, it is impossible to tag the sample with at least 2 and fewer than the number of polynucleotides that map to the mappable base position (*i.e.*, one polynucleotide), as required by the challenged claims. EX1001, 41:42-47; *see also* EX2025, ¶¶74-75.

Although not in the petition, to the extent that Petitioner attempts to newly argue that additional copies of the genome would be analyzed, the proposed 65,536 n-mers would need to be used to tag a greater number of genome copies. Extending Petitioner's contrived logic, at least 65,537 copies of the haploid human genome would be required for it to represent non-uniquely tagged according to the claims. [2]

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[2] To be clear, this example is for illustrative purposes. Dr. Quackenbush explains that a vastly greater number of genome copies would be required to even approximate the number of tags available. As discussed elsewhere herein,

That is, there would need to be more copies of the human genome than tags. EX1001, 41:42-47. If 65,537 haploid copies of the human nuclear genome were sequenced, this would represent a target size of about 10^{14} base pairs (*i.e.*, 65,537 copies x 3,000,000,000 base pairs). As Dr. Quakenbush explains, such a scenario would require an extraordinary amount of sequencing, far in excess of what is typically conducted even in present day experiments. *See also* EX2025, ¶¶76-78.

Such a scenario also runs contrary to Schmitt. Schmitt does not disclose sequencing targets as large as the entire 3 billion base pair haploid human genome, much less 65,537 copies of said genome. Instead, Schmitt discloses applying DCS to much smaller targets. EX1011, 24:7-11 ("Current experiments are being performed on vastly smaller target DNA molecules (ranging from ~300 bp to ~20 kb in size). Use of smaller DNA targets will allow for much greater sequencing

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Schmitt's method involves discarding large portions of sample and data, and employs a highly inefficient tagging scheme. Schmitt's process of random fragmentation and discarding most polynucleotides alone would result in only a minute fraction of genome copies in a sample producing a polynucleotide that maps to a given mappable base position. EX1011, 22:43-46 (describing random fragmentation and discarding polynucleotides outside the optimal range of 200-500bp). *See also* EX2025, ¶78.

depth, and far more accurate assessment of the background mutation rate of the assay."). *See also* EX2025, ¶79.

Moreover, in a later publication by the inventors of the Schmitt patent, the DCS method was characterized as being "prohibitively expensive" for genome targets in excess of 1-2 Mbp. EX1057 at 4. That is, DCS is prohibitively expensive for targets three orders of magnitude smaller than a one copy of the haploid human genome. *See also* EX2025, ¶80.

iii. *Schmitt exclusively teaches unique tagging methods*

The purported invention of the Schmitt patent is expressly identified as methods involving unique single molecule identifier ("SMI") or n-mer tags, which are critical to Schmitt's grouping of amplicons and consensus sequence construction. This is repeatedly expressed throughout the Schmitt patent. *E.g.*, EX1011, 4:6-7 ("sequence reads … sharing a ***unique set of SMI tags*** are grouped into paired families"), 3:47-49 ("Because every adaptor contains a ***unique***, double-stranded, complementary n-mer random tag on each end…"), 6:59-63 ("The first and/or second degenerate or semi-degenerate nucleotide n-mer sequences may be any suitable length to produce a ***sufficiently large number of unique tags*** to label a set of sheared DNA fragments from a segment of DNA."), 18:36-41 ("Because the use of SMI tags … allows every molecule to be ***uniquely labeled*** prior to PCR duplication, true PCR duplicates may be unambiguously identified by virtue of

having a common (i.e., the same or identical) SMI sequence."), 21:59-61 ("The sequences of ***uniquely tagged PCR duplicates*** are subsequently compared in order to create a PCR consensus sequence."). *See also* EX2025, ¶81.

Nevertheless, the petition materials assert that n-mer tags used in the "hybrid method" of Schmitt are not "unique" because that embodiment necessarily employs fewer barcodes than total polynucleotide fragments. Pet. 43-44 (citing EX1011, 9:1-13, Figure 4); Paper 12 at 18-19. This argument is flawed for a number of reasons. *See also* EX2025, ¶82.

First, as set forth above, Petitioner's argument fails as a threshold matter for ignoring the undisputed express construction of "non-uniquely tagged," as recited in the challenged claims. In particular, the petition materials erroneously address an entire population of polynucleotides in a sample, rather than "polynucleotides that map to the mappable base position." *See also* EX2025, ¶83.

Second, regardless of the failure to adequately address the challenged claims, Petitioner's argument improperly relies on circular reasoning, making assumptions not supported by the cited reference. In particular, the petition materials assume a combination of more fragments in a sample (i.e., millions of fragments) than SMI tags (i.e., 65,536 tags) in order to conclude that the number of fragments is greater than the number of tags. That is, Petitioner assumes the very conclusion it seeks to reach. Such flawed logic falls short of the rational underpinnings required to

support an assertion of obviousness. *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006); see also *KSR*, 550 U.S. at 418, 82 USPQ2d at 1396 (quoting Federal Circuit statement with approval). Moreover, petitioner's assumptions that are unsupported to the actual content of the cited reference. *See also* EX2025, ¶84.

Initially, does Schmitt describe sequencing and analysis of the vast quantities of polynucleotides of an entire human genome as Petitioner assumes in its hypothetical. For example, Schmitt discloses applying DCS to much smaller targets — "ranging from ~300 bp to ~20 kb in size." EX1011, 24:7-11. Assuming such targets are sheared into 200 bp fragments, *id.*, 19:65-66, a target of 300 bp to 20,000 bp would at best generate between 1 and 100 fragments. That is, far fewer fragments than "millions of fragments" and certainly less than tags generated by a 4-mer. *See also* EX2025, ¶85.

Additionally, as explained by Dr. Quackenbush, Schmitt never instructs that the number of n-mer tags should be less than the polynucleotides in a sample. Schmitt states just the opposite. That is, where Schmitt does relate the number of barcodes to polynucleotide fragments in a sample it instructs that the barcodes should be in excess of the sample fragments. See e.g., EX1011, 3:47-53 ("Because every adaptor contains a unique, double-stranded, complimentary n-mer random tag on each end (n-mer=12 bp according to one embodiment), every DNA

fragment becomes labeled with two distinct SMI sequences…); *Id.*, at 6:59-62 ("The first and/or second degenerate or semi-degenerate nucleotide n-mer sequences may be any suitable length to produce a sufficiently large number of unique tags to label a set of sheared DNA fragments from a segment of DNA."). As Dr. Quackenbush explains, the instructions of Schmitt in this regard are in the general context of the DCS method employing n-mer or SMI tags.[3] There is no instruction to the contrary. *See also* EX2025, ¶86.

Petitioner's reference to an embodiment that does not use any n-mer tag does not dictate any other conclusion. Pet. 43 (citing EX1011, 9:1-4 as providing an embodiment that "does not strictly require the use of an SMI tag, as the sheared ends can be used as identifiers to differentiate unique individual molecules from PCR duplicates'").[4] While Schmitt mentions such a possibility, it then goes on to

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[3] The petition acknowledges that "Schmitt sometimes refers to the SMI sequences as 'n-mer' sequences." Pet. 43 (citing EX1011, 6:46-66; EX1002, ¶¶125, 128); see also, Institution Decision, 18 ("Schmitt also refers to the sequences of the SMI adaptors as "n-mer" sequences.").

[4] Petitioner does not, as it could not, assert that such a tag-free embodiment meets the requirements of the challenged claims. Certainly, an embodiment that

identify such an approach as limited and advocate for inclusion of an SMI tag. EX1011, 9:7-10 ("there are a limited number of shear points flanking any given genomic position and thus the power to sequence deeply is increased via inclusion of an SMI tag."). *See also* EX2025, ¶87.

As indicated above, in the embodiments where Schmitt does include an n-mer/SMI tag, Schmitt instructs using a sufficiently large number of tags. The petition acknowledges that the "hybrid" method includes n-mer tags. Pet. 44 ("the hybrid method us[es] a combination of sheared ends and a shorter ***n-mer*** tag.")(quoting EX1011, 9:1-13); see also EX2020, ¶196 (Dr. Gabriel testifying that the "hybrid approach relies on a combination of ***barcodes*** and sheared ends of the fragments themselves"). Limiting n-mer tags as suggested in the petition materials would be contrary to the express instruction of Schmitt, and counterintuitive considering that Schmitt critically relies on unique tagging in order to group amplicons for building consensus sequences. *See also* EX2025, ¶89.

Finally, the petition materials assert that "Schmitt's 'hybrid method' is nearly identical to an embodiment of the '822 patent." Pet. 45; EX1002, ¶130 (same). This is incorrect. *See also* EX2025, ¶90.

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does not tag polynucleotides at all is outside the scope of claims requiring "non-uniquely **tagged** parent polynucleotides." *See also* EX2025, ¶88.

As explained above, the '822 patent discloses a non-unique tagging method that relies on both mappable base position and identifiers (e.g., barcodes) to distinguish parent polynucleotide molecules. The '822 patent discloses that a much smaller number of tags may be used such that at least some of the cognates— that is, polynucleotide molecules that map to the same start and stop position in the reference sequence but are derived from different copies of a genome — are distinguishable. EX1001, 40:4-8, 41:25-28; *see also* Section V; EX2025, ¶91.

Schmitt's hybrid method takes a fundamentally different approach that critically relies on random shearing of cellular DNA. EX1011, 9:9-13; EX2020, ¶196 (Dr. Gabriel testifying that the "hybrid approach ***relies on*** a combination of barcodes and ***sheared ends*** of the fragments themselves"); *see also* EX2025, ¶92.

The methods of the '822 patent do not and cannot employ the shearing method of Schmitt. As Dr. Quackenbush and Dr. Shendure explain, the random shearing method of Schmitt (i.e., acoustic shearing) is incompatible with cfDNA. Even Petitioner's expert witness, Dr. Gabriel, admits that shearing is not appropriate for cfDNA. EX2022, 105:14-20 ("Q. You would take [acoustical shearing] out [of Schmitt] to reach the claimed invention of the '822 patent, correct? … A. As I discussed many times in these reports, I would not use mechanical or acoustic shearing of cell-free DNA."); *see also* EX2025, ¶93.

What the petition materials point to as teaching "non-unique tagging" is both (1) unique tagging and (2) entirely dissimilar to the method claimed in the '822 patent.

B. Neither Schmitt nor any combination of references teaches a method for converting cell-free DNA into tagged parent polynucleotides

The claims of the '822 patent require "converting the population of cfDNA molecules into a population of non-uniquely tagged parent polynucleotides." The petition focuses entirely on the "non-uniquely tagged" element of claim 1 and fails to discuss, much less establish, that Schmitt or any combination of references teaches converting cfDNA molecules into tagged parent polynucleotides. *See* Pet. 42-46; *see also* EX2025, ¶94.

The petition relies entirely on the hybrid method of Schmitt for the "non-uniquely tagged" element of claim 1, *see* Section VII.A., but this method is directed exclusively to cellular DNA. Specifically, the hybrid method critically relies on sheared cellular DNA fragments. EX1011, 9:9-13. Dr. Gabriel concedes Schmitt's reliance on shearing. EX2020, ¶196 ("[A] person of ordinary skill in the art would understand the description of the hybrid approach in Schmitt and Schmitt 2012 to teach non-unique tagging because the hybrid approach relies on a combination of barcodes and sheared ends …"). *See also* EX2025, ¶93

Furthermore, as Dr. Quackenbush and Dr. Shendure explain, and as Dr. Gabriel also concedes, shearing is incompatible with cfDNA samples. Shearing of

cfDNA would result significant destruction of fragments and loss of sample — a result that could not be tolerated when working with the minute quantities of cfDNA that may reasonably be obtained from a patient. *See also* EX2025, ¶95; EX2023, ¶33.

Moreover, according to Dr. Quackenbush and Dr. Shendure, sheared cellular DNA and cfDNA are not equivalent. Sheared cellular DNA is randomly fragmented, which as Dr. Gabriel points out, provides information that may be used to uniquely identify fragments. EX2021, 238:6-11 ("Q. And what's the reason that the hybrid approach relies on sheared ends? A. The sheared ends add to the sort of information content of the UID and make that molecular identifier more unique, more identifiable."). Cell-free DNA, as Dr. Gabriel further acknowledges, displays a fragmentation pattern as a result of being produced enzymatically in cells. EX2021, 23:23-24:3, 24:13-19, 24:23-25, 25:5-26:4. As a result, cfDNA has a much lower diversity of sequences at the termini as compared to acoustically sheared cellular DNA.

For at least these reasons, Dr. Quackenbush explains that a POSA would not have considered Schmitt to teach converting cfDNA into tagged parent polynucleotides. Moreover, this deficiency is not remedied by Fan or Forshew — nor does the petition assert that it is. Fan simply does not tag parent

polynucleotides. EX1048. To the extent that Forshew discloses tagging, it is to place sample barcodes on PCR amplicons. EX1004; *see also* EX2025, ¶¶95-101.

C. The petition materials do not establish that a skilled artisan would have wanted to combine Schmitt with either Fan or Forshew

As the Federal Circuit has explained a conclusion of obviousness requires establishing "a motivation to make the combination." *In re Stepan Co.*, 868 F.3d 1342, 1346 n.1 (Fed. Cir. 2017). The petition wholly fails to establish that a POSA would have been motivated to combine Schmitt with either Fan or Forshew. As a result, Petitioner's challenge may be rejected for this reason alone.

In place of motivation to make the combination, Petitioner offers only mischaracterizations and flawed logic. For example, the petition asserts that Schmitt "expressly" contemplates application of DCS for prenatal screening for fetal aneuploidy. Pet. 36 (citing EX1011, 1:42-43) ("Clinical applications, such prenatal screening for fetal aneuploidy [9, 10] …"). However, according to Dr. Shendure and Dr. Quackenbush, a POSA would not understand this passage from the background section to be contemplating application of DCS to fetal aneuploidy detection. Rather, Schmitt is merely mentioning in some introductory remarks that different techniques employing DNA sequencing have been used by others to detect fetal aneuploidy. *See also* EX2025, ¶¶102-110; EX2023, ¶20.

Moreover, as Dr. Quackenbush explains, there would be no good reason to apply Schmitt's DCS method to detecting fetal aneuploidy, such as disclosed by

Fan. Schmitt discloses that it is desirable to use error correction to detect "rare variants" such as single nucleotide variants in "heterogeneous populations." EX1011, 2:56-62; *see also* EX2025, ¶106. But as Dr. Gabriel concedes, rare variants are not detected in aneuploidy assays. EX2021, 158:19-22 ("Would you characterize the aneuploidy in the cell-free DNA of a pregnant woman, would you characterize that as a rare mutation? A. No. I would not characterize it that way."). No sequence information (*e.g.*, identification of single nucleotide variants) is provided by Fan's method. Rather, Fan detects fetal aneuploidy by counting mapped reads and determining if there is an excess number of reads relative to a reference data set for a given chromosome. EX1048 at 16267-68 ("Detection of Fetal Aneuploidy); EX2021, 57:3-10 ("Q. And that's because [Fan and Chiu are] not looking for like individual point mutations. They just need to have adequate enough sequence information to say where the sequence read map to in the genome. … Q. Is that right? A. In these publications, that's what they're doing.").

Consequently, application of Schmitt's DCS method to aneuploidy detection would needlessly increase the expense and complexity of the method. *E.g.*, EX1057 at 4 ("DS is a uniquely sensitive method that is capable of detecting and quantifying mutations that occur at extremely low frequencies; however, this ability comes at a cost. Owing to the method's reliance on sequencing multiple PCR duplicates of both DNA strands, DS requires much larger sequencing

capacity than conventional NGS to produce a given depth of sequencing."). *See also* EX2025, ¶107.

The petition further asserts that "a POSA would have been motivated to combine the teachings of Schmitt and Forshew because both disclose methods of 'deep sequencing' to detect low frequency mutations." Pet. 37 (citing EX1002, ¶109). The Board also stated that "that Schmitt's DCS method was a reasonable substitute for the deep sequencing techniques used in the prior art — such as those techniques Fan and Forshew used for prenatal cfDNA screening and for tumor cfDNA screening." Paper 12 at 26. As an initial matter, Schmitt's DCS method is not a "deep sequencing" technique but rather a method for correcting errors in NGS reads. EX1011, Abstract. That Schmitt and Forshew each use NGS as part of their disclosed methodology is not evidence that a POSA would have wanted to — or could have —combined these methods. *In re Magnum Oil Tools Int'l, Ltd.*, 829 F.3d 1364, 1379 (Fed. Cir. 2016) (merely "noting surface similarities between the [asserted] references" is insufficient to demonstrates motivation to combine; "[t]hese surface similarities do not control the obviousness analysis because they are not directed to the relevant features of the claimed invention"). At most, Schmitt, Fan, and Forshew may be analogous art. But it does not suggest a reason for specifically applying Schmitt to detection of low frequency mutations in

cfDNA as is required to reach a conclusion of obviousness. *Stepan*, F.3d at 1342, 1346 n.1; *see also* EX2025, ¶¶108-110.

Moreover, as Dr. Quackenbush and Dr. Shendure explain, a POSA would not have found Schmitt applicable to cfDNA. *See* Section VII.E.

D. The petition materials do not establish a reasonable expectation of success in applying Schmitt to cfDNA

As with motivation to combine, reasonable expectation of success is a required element of any obviousness case. *Stepan*, F.3d at 1342, 1346 n.1. Despite having a section directed to "reasonable expectation," the petition avoids the critical issue — that is, why a POSA would have expected Schmitt to work with cfDNA. Instead of addressing this issue, Petitioner offers assertions that are either not supported, irrelevant, or wrong. *See also* EX2025, ¶111.

Petitioner repeatedly fails to address whether Schmitt could be applied to cfDNA with a reasonable expectation of success. For example, the petition asserts that a "person of skill in the art would have known that next generation sequencing technologies, including barcode tag-based approaches to sequencing, had been used successfully to detect rare mutations in both cellular and cell-free DNA." EX1002, ¶118 (citing EX1049; EX1059 at 2); *see also* Pet. 39-40. This sidesteps the issue. Neither cited exhibit uses the Schmitt method. Even if other methods using NGS had been applied to cfDNA, it does not establish that the different Schmitt method is applicable to cfDNA. Furthermore, exhibit 1049 does not even

disclose methods directed to cfDNA, instead it discloses methods for barcoding amplicons derived from cellular DNA. Exhibit 1059 does not disclose detection of rare mutations, rather it describes a method for detecting fetal aneuploidy by counting sequencing reads. EX2021, 158:19-22. That is, neither reference establishes that Schmitt could be applied to cfDNA with a reasonable expectation of success. *See also* EX2025, ¶112.

The petition further asserts that "a person of ordinary skill in the art reasonably would have expected the DCS method, as described by both Schmitt and Schmitt 2012, to be sensitive enough to detect rare mutations irrespective of the source of the DNA to be sequenced." EX1002, ¶115; *see also* Pet. 38-39 (similar). In support of this assertion, the petition materials point exclusively to application of DCS to cellular samples. EX1047 at 14511 (identifying mutation in plasmid DNA); EX1011, 30:7-10 (same); EX1056 at 14290 (same). Using DCS to identify mutation in plasmid DNA does not address the applicability of DCS to cfDNA. *See also* EX2025, ¶¶113-114.

Other arguments made in the petition are simply indecipherable. For example, the petition asserts that "the DCS method does not require substantive modification of existing sequencing workflows and can serve as a general technique for next generation sequencing." EX1002, ¶114 (citing EX1011, 18:44-61); *see also* Pet. 54-55. It is not clear what Petitioner means by "general technique"

or what this has to do with the applicability of Schmitt to cfDNA. Dr. Quackenbush reviewed the petition materials but was unable to discern any relevance to this assertion.

The petition also relies on impermissible hindsight to address expectation of success. For example, the petition materials assert that "the ***claimed methods*** are equally applicable to cell-free DNA and cellular DNA." Pet. 39; *see also* EX1002, ¶118 (similar). That the claimed methods—which are not the same as the Schmitt method—may be applied to both cfDNA and cellular DNA is not an indication that Schmitt would be applicable to cfDNA. The petition also asserts that the claims of the '822 patent recite only well-known sample preparation, sequencing and analysis steps" and "Schmitt discloses every one of these same basic steps, namely generating sequence reads by tagging fragmented DNA with barcodes, amplifying the tagged DNA, and sequencing the amplified DNA." EX1002, ¶116; *see also* Pet. 39; *Princeton Biochemicals, Inc. v. Beckman Coulter, Inc.*, 411 F.3d 1332, 1337 (Fed. Cir. 2005) ("using the invention as a roadmap" represents impermissible hindsight). Not only does this argument represent impermissible hindsight, but Petitioner never establishes that the '822 patent recites only "well-known" elements. Indeed, this argument is undercut by Petitioner's failure to identify multiple claim elements in the prior art. *See* Sections VII.A. and VII.B; *see also* EX2025, ¶¶115-117.

The petition relies on the unsubstantiated assertion that "[a] person of ordinary skill in the art also would have recognized that the tagging, amplifying, and sequencing steps described by Schmitt would operate in the same or an equivalent manner whether one was starting with cellular DNA or cell-free DNA." Pet. 39; *see also* EX1002, ¶118 (similar). Not so, even Dr. Gabriel conceded the falsity of this assertion during cross examination. EX2021, 158:4-12; EX2022, 105:11-20 ("11 Q. I'm asking about the mechanical and acoustical shearing in Schmitt. A. Yes. Q. You would take that out to reach the claimed invention of the '822 patent, correct? …18 A. As I discussed many times in these reports, I would not use mechanical or acoustic shearing of cell-free DNA.").

The petition materials falsely assert that "[t]he size of the cellular genomic DNA fragments that Schmitt sequenced and analyzed is similar to the naturally fragmented size of cfDNA." Pet. 39 (citing EX1002, ¶118). Schmitt discloses precisely the opposite — that is, discarding fragments of similar size to cfDNA. Schmitt describes the optimal range of fragment sizes for DCS is 200-500 bp. EX1011, 19:65-66, 22:43-46. Petitioner does not dispute this fact. Pet. 44 (citing EX1011, 22:43-46; EX1002, ¶127) ("Schmitt teaches selection of fragments in the optimal range of 200-500 base pairs …."). Schmitt describes removing fragments that are larger than 500 bp and smaller than 200 base pairs prior to tagging fragments. EX1011, 19:65-20:15 (describing "size selecting" fragments in the

range of 200-500 bp prior to attaching tags), 22:43-54 (same). Petitioner tries to obscure this fact by only citing that fragments greater than 500 bp are removed. Petitioner fails to inform the Board that Schmitt also discards fragments less than 200 bp — that is, fragments the size of cfDNA. That is, Schmitt does not tag, much less analyze fragments, similar to the naturally fragmented size of cfDNA. *See also* EX2025, ¶118.

The petition concedes that the shearing step of Schmitt is necessary to make fragments that are "compatible with amplification and NGS sequencing." EX1002, ¶118; *see also* Pet. 39-40. But this is not the only purpose of the shearing step of Schmitt. Specifically, Schmitt discloses that the hybrid method relies on randomly sheared ends. EX1011, 9:9-13; *see also id.*, 25:64-65 ("The DNA was randomly sheared by the Covaris AFA system …"), 17:41-45 ("using the random shear points of DNA as identifiers"). Dr. Gabriel acknowledges that the hybrid method relies on these random shear points of cellular DNA. EX2020, ¶196; EX2021, 238:6-11 ("Q. And what's the reason that the hybrid approach relies on sheared ends? A. The sheared ends add to the sort of information content of the UID and make that molecular identifier more unique, more identifiable."). *See also* EX2025, ¶119.

Finally, Petitioner falsely claims that Schmitt has been applied to cfDNA, citing Krimmel (EX1060). Pet. 40. Krimmel has no such disclosure. As Dr.

Quackenbush explains, Krimmel (EX1060) **does not** disclose applying Schmitt's method to cfDNA. Krimmel applies DCS to cellular DNA. EX1060 at Abstract ("Our aim was to determine whether duplex sequencing could detect extremely rare ***cancer cells*** present in peritoneal fluid from women with high-grade serous ovarian carcinomas (HGSOCs)."), ("Our results demonstrate the ability of duplex sequencing to detect rare ***cancer cells*** …"), 6005 ("We reasoned that peritoneal fluid is an optimal clinical biopsy for high sensitivity early detection of ovarian cancer because HGSOC disseminates early and preferentially to the peritoneal cavity, suggesting that ***cancer cells*** may be more abundant in peritoneal fluid…"), 6006 ("Our goal was to provide proof of principle of the ability of duplex sequencing to detect very rare ***cancer cells*** …"), ("Peritoneal fluid was centrifuged, and DNA was extracted from the ***cell pellet***, obtaining an average of 11.9 μg of DNA."), 6009 ("[D]uplex sequencing demonstrated here could also be applicable to the identification of ***cancer cells*** from other cancer types …"), 6010 ("DNA was extracted from peripheral whole blood and peritoneal fluid sample ***cell pellets*** …"). *See also* EX2025, ¶¶120-121.

That Schmitt's DCS method is inefficient and poorly suited for cfDNA samples, is well documented in the literature, including by the inventors of DCS. For example, Perakis, et al. concluded that "prospects of success" in applying DCS to cfDNA were limited:

> Although [Schmitt] used this approach for selective enrichment of small genomic regions, the original protocol has not yet been used with ctDNA. Prospects of success are limited since the method is relatively inefficient when limited amounts of input DNA—as it is most likely the case for cfDNA—are used.

EX2002 at 102; *see also* EX2025, ¶122.

Newman, et al. came to the same conclusion:

> Although duplex barcoding achieves better error suppression than single-stranded barcoding methods, it is relatively inefficient and thus suboptimal for the limited cfDNA quantities obtainable in a clinical setting.

EX2039 at 548; *see also* EX2025, ¶123.

Kamps-Hughes, et al. further commented on the necessity for "extremely high" DNA input amounts:

> Molecular barcoding has shown promise in drastically lowering the limit of detection for variants in NGS data. However, the proof of principle experiments demonstrating high accuracy at ultralow allele frequencies have required extremely high read depths and DNA inputs while demonstrating efficacy only over very small genomic regions.

EX2040 at 2; *see also* EX2025, ¶124.

Wang, et al., addressed the "critical losses" of template DNA:

> For instance, Duplex Sequencing creates high quality DCSs with exceedingly low error rates but at the expense of inefficient processes leading to critical losses of template DNA molecules.

EX2041 at 10. The issue of sample loss was even addressed by the inventors of DCS:

> [I]t is most convenient to start with excess amounts of DNA to allow for easy quantification of the DNA throughout the initial sample

preparation steps and to account for expected sample loss at the enzymatic and purification steps.

EX1057 at 5; *see also* EX2025, ¶125.

That the DCS method disclosed by the Schmitt references is poorly suited for cfDNA samples is beyond reasonable dispute. *See also* EX2025, ¶126.

E. Numerous aspects of Schmitt would have been recognized as incompatible with cfDNA

As set forth above, Petitioner fails to establish motivation to combine with a reasonable expectation of success. *See* Sections VII.C. and D. While it is not Patent Owner's burden to prove no motivation with an expectation of success, that is the only reasonable conclusion in view of the evidence, testimony of multiple witnesses, and scientific literature (see above) characterizing Schmitt as incompatible with cfDNA analysis. *See also* EX2023, ¶¶16-37; EX2025, ¶127-138.

As an initial matter, Schmitt applies the DCS method exclusively to samples of cellular DNA. EX1011, Abstract. Cellular DNA samples are discussed throughout Schmitt and each of the examples addresses cellular samples (*e.g.*, "normal human colonic mucosa," "human mitochondrial DNA," bacterial cells, and yeast cells). EX1011, 23:4-5, 4:55-56, 24:64-65, 25:63-64, 5:12, 27:37-38. Schmitt does not even mention applying DCS to cfDNA, let alone provide any corresponding analysis. *See also* EX2023, ¶¶18.19.

Furthermore, as explained by Dr. Quackenbush and Dr. Shendure (and conceded by Dr. Gabriel) a POSA would have considered Schmitt incompatible with cfDNA analysis. As explained in further detail below, Schmitt requires large quantities of input DNA that are simply not available when working with cfDNA due to the minute quantities that are reasonably obtained from patients. Additionally, there are several critical aspects of the Schmitt method (*e.g.*, random shearing, optimal fragment size, randomly generated tags, numerous processing steps) that would be recognized as being incompatible with cfDNA sample analysis. *See also* EX2023, ¶¶21-22.

As a threshold matter, Schmitt characterizes the polynucleotide fragments of the size that would be found in cfDNA samples as being sub-optimal for its method. For example, Schmitt instructs selection of fragments in the optimal range of 200-500 base pairs. EX1011, 19:65-66 (identifying "the optimal range of ~200-500 bp" for its DCS method), 22:43-46; *see also* EX2023, ¶¶23.

The average size of cfDNA fragments, however, is about 140 to 170 base pairs—which is outside of Schmitt's fragment size range. Schmitt, in fact, instructs that fragments of less than 200 base pairs (*e.g.*, fragments about 140 to 170 base pairs) should be discarded in the sample preparation steps of the DCS method. EX1011, 19:63-20:4 ("DNA fragments larger than the optimal range of ~200-500 bp were removed…."), ("this step allows fragments of approximately 200 bp or

greater to bind to the beads."), 22:43-46 (describing "size-selection for fragments in the range of ~200-500 bp by size-selective binding to Ampure XP beads."); *see also* Pet. 40 (citing EX1004, 1). Discarding fragments outside the optimal range of about 200-500 base pairs is not compatible with cfDNA analysis as it would result in discarding a large portion of a cfDNA sample. *See also* EX2023, ¶¶23-24

Schmitt instructs additional steps that discard sample and data during processing and analysis. EX1011, 20:42-43 (describing "filtering out reads that lack the expected fixed sequence at positions 13-17."), 20:47-50 ("The first 4 nucleotides located following the adaptor sequence were also removed…"), 20:53-55 ("Sequencing positions were discounted if the consensus group covering that position consisted of fewer than 3 members…"), 20:65 ("Discard reads that do not have the 5 nt fixed reference…"), 21:4-18 ("Discard SMI's with inadequate complexity…. Remove the 5 nt fixed reference sequence…. Trim an additional 4 nt from the 5' ends of each read pair (sites of error prone end repair) …. Only score positions with identical sequence among both DCS partners."). *See also* EX2023, ¶¶25.

Turning to the SMI tags, this aspect of Schmitt represents an additional source of data loss. Schmitt critically relies on randomly generated SMI tags—but such tags further result in significant data loss in Schmitt's method. Schmitt's method groups reads into families based on the SMI tags. Because the SMI tags (as

well as sheared ends) are randomly generated, the tag sequences are not known in advance and errors (*e.g.*, sequencing error, PCR misincorporation, etc.) cannot be identified. As such, sequence reads with errors in the tag region could not be grouped according to Schmitt's method and must be discarded. As explained in further detail below, such errors are significant in Schmitt as even a modestly sized 12-mer SMI would require discarding 1 out of every 4 sequence reads. *See also* EX2023, ¶¶26.

As Schmitt explains, the SMI tags comprise degenerate n-mers — that is, randomly generated sequences. *E.g.*, EX1011, 6:51-53, 6:59-63 ("The first and/or second degenerate or semi-degenerate nucleotide n-mer sequences may be any suitable length to produce a sufficiently large number of unique tags to label a set of sheared DNA fragments from a segment of DNA."), 6:66-7:5, 9:9-13 ("A hybrid method using a combination of sheared ends and a shorter n-mer tag (such as 1 or 2 or 3 or 4 or more degenerate or semi-degenerate bases) in the adaptor may also serve as unique molecular identifiers."). Schmitt also discloses that approximately 1% of bases are incorrectly identified by NGS. EX1011, 1:64-2:4; *see also* Pet. 34. These errors are incorporated throughout the polynucleotide fragment, including in the SMI tag and shear points. Since every fragment in Schmitt includes two tags (one at each end), even a relatively low error rate of 1% will result in error in about every fourth sequence read of Schmitt. *See also* EX2023, ¶¶27-28.

Base misincorporation errors in the SMI tag (or shear points) cannot be corrected. Rather, an error in the SMI tags would prevent the grouping of the polynucleotide into the appropriate family as required by Schmitt. *E.g.*, EX1011, Fig. 3, 4:4-29. As a result, polynucleotides with sequence errors in the SMI tag cannot be used for error correction and must be discarded. *See also* EX2023, ¶¶29.

In view of inefficiencies in the method and steps requiring discarding significant amounts of sample and data, Schmitt requires large amounts of cellular DNA in its assays. For example, Schmitt discloses using 3 µg of human DNA. EX1011, 19:18-19, 19:58. While cellular DNA might be available in such large quantities, cfDNA is not. For instance, 1 mL of human serum typically contains only about 10 ng of cfDNA. That is, the amount of cfDNA in 1 mL is about 3-orders of magnitude less than what Schmitt discloses is used for DCS. As Dr. Shendure explains, it is simply not feasible to obtain cfDNA in microgram quantities from patients. *See also* EX2023, ¶¶30-31.

The difference in the amount of DNA between what is used in the Schmitt method and what is practicable to obtain from a patient is not trivial. Adapting Schmitt to apply to cfDNA is not just a matter applying the technique to a more dilute DNA sample but would require fundamental modification and redesign of the DCS method. Moreover, enzymatic steps (*e.g.*, end repair, T-tailing, ligation,

EX1011, 19:58-20:15) work less efficiently with dilute samples. *See also* EX2023, ¶¶32.

Additional aspects of Schmitt's method are not compatible with cfDNA analysis. For example, Schmitt's method also relies on generating sheared ends. EX1011, 9:9-13, 19:58-60. Dr. Gabriel acknowledges that the hybrid method relies on these random shear points of cellular DNA. EX2020, ¶196; EX2021, 238:6-11 ("Q. And what's the reason that the hybrid approach relies on sheared ends? A. The sheared ends add to the sort of information content of the UID and make that molecular identifier more unique, more identifiable."). In particular, Schmitt instructs generating sheared ends by acoustic shearing of cellular DNA. Acoustic shearing results in a random distribution of sequences at the ends of polynucleotide molecules. The shearing method of Schmitt is not compatible with cfDNA. Dr. Gabriel admits that shearing would not be used with cfDNA. EX2021, 158:4-12 ("Q. Okay. And the method in Schmitt uses shearing of cell-free DNA, correct? A. Not shearing of cell-free DNA. Q. Excuse me, that's right. It uses shearing of genomic DNA, correct? A. Correct. Q. And you wouldn't apply that shearing technique for cell-free DNA, right? A. I would not."); EX2022, 105:11-20 ("11 Q. I'm asking about the mechanical and acoustical shearing in Schmitt. A. Yes. Q. You would take that out to reach the claimed invention of the '822 patent,

correct? …18 A. As I discussed many times in these reports, I would not use mechanical or acoustic shearing of cell-free DNA."). *See also* EX2023, ¶¶33-34.

Cell-free DNA is already fragmented and has a peak size of around 160 nucleotides. Further reduction in size, such as by shearing, would result significant destruction of fragments and loss of sample that is already present in only minute quantities. Moreover, the even smaller fragments that would be difficult to isolate and sequence.

Moreover, while cfDNA is fragmented, the fragments do not resemble those generated by Schmitt's shearing process. As indicated above, cfDNA fragments are smaller than the optimally sized fragments (*i.e.*, ~200-500 base pairs) generated and isolated in Schmitt's method, and reflective of fragments Schmitt instructs should be discarded. Additionally, cfDNA is not randomly fragmented as would be generated by Schmitt's shearing process, but instead represent a more patterned fragmentation. Cell-free DNA is produced enzymatically in cells which results in a much lower diversity of sequences at the termini as compared to acoustically sheared cellular DNA. *See also* EX2023, ¶¶35.

Dr. Gabriel acknowledges that cfDNA is not randomly fragmented, but rather displays a pattern. EX2021, 23:23-24:3 (describing cfDNA as having a "fragmentation pattern" resulting from cleavage in apoptotic cells), 24:13-19 (describing fragmentation pattern as a result of "circling of DNA around histones"),

24:23-25 (describing pattern as "tissue-specific and gene specific and gene-expression-specific"), 25:5-26:4 ("Q. I see. So the transcription sites are the regions of the genome that are not protected by the nucleosomes and so they're exposed for cutting? A. There are regions upstream of the transcription start sites, which there will be nucleosomes bound and which will result in areas where DNA can be available for some fragmentation or cutting."). *See also* EX2023, ¶¶36.

Schmitt critically relies on high diversity of identifiers in order to group PCR amplicons in families and build a consensus sequence. EX2021, 238:6-11 ("Q. And what's the reason that the hybrid approach relies on sheared ends? A. The sheared ends add to the sort of information content of the UID and make that molecular identifier more unique, more identifiable."). Accordingly, not only is shearing incompatible with cfDNA, low diversity fragmentation is counter to the objective of Schmitt as more sequence data will be lost for inability to distinguish individual polynucleotide molecules. *E.g.*, EX1011, 21:4 ("Discard SMIs with inadequate complexity"), 18:28-32 ("When multiple molecules exhibit identical shear points, all but one of the molecules are discarded from analysis under the assumption that the molecules represent multiple PCR copies of the same starting molecule."). *See also* EX2023, ¶¶36-37.

As Dr. Shendure and Dr. Quackenbush explain, for at least these reasons, a POSA would not view Schmitt as teaching, or even contemplating, application of

its methods to cfDNA. In fact, a POSA would have considered Schmitt poorly suited for cfDNA analysis for numerous reasons. *See also* EX2023, ¶¶37

F. The petition materials do not establish that dependent claims 2-13 and 17-20 are unpatentable

Claims 2-13 and 17-20 are not obvious for the same reasons identified above with respect to claim 1 for each of the grounds of challenge. Additional reasons why some of claims 2-13 and 17-20 are not unpatentable are discussed below. EX2025 ¶¶140-155.

i. *Claim 2 is not unpatentable*

Claim 2 further requires "detecting, at one or more loci, at least one single nucleotide variant, at least one gene fusion and at least one copy number variant."

The petition materials assert that Schmitt "explicitly" teaches that DCS can detect copy number variation ("CNV"). Pet. 57 (citing EX1002, ¶157). But the petition materials fail to point to any disclosure of detection of CNVs — because there is none. Instead, the petition materials point to disclosure of "single molecule counting for accurate determination of DNA or RNA copy number." Pet. 58 (citing EX1011, 18:3-21; EX1002, ¶158).

The petition materials appear to be suggesting that the single molecule counting method of Schmitt could be used in an analogous manner to the unrelated disclosure of Kivioja used to detect whole chromosome aneuploidy. EX1002,

¶¶158-159. Even if true, this does not establish that Schmitt teaches CNV detection explicitly or otherwise.

The petition materials also assert that "[t]he POSA would have understood that different types of mutations could be identified from the same data set." Pet. 58 (citing EX1002, ¶¶160-161; EX1005, Figure 3; EX1067; EX1068). What these "different types" of mutations are is unexplained. Furthermore, none of the references cited by the petition materials disclose application of the Schmitt method to detection of "at least one single nucleotide variant, at least one gene fusion and at least one copy number variant."

ii. ***Claim 5 is not unpatentable***

Claim 5 further requires "making the base call comprises voting, averaging, maximum a posteriori or maximum likelihood detection, dynamic programming, Bayesian methods, hidden Markov methods or support vector machine methods."

The petition materials point to no disclosure in Schmitt of using voting, averaging, maximum a posteriori or maximum likelihood detection, dynamic programming, Bayesian methods, hidden Markov methods or support vector machine methods to make base calls. Instead, Petitioner merely states these methods were known in the art. EX1001, ¶167. Nowhere is it explained why a person of ordinary skill in the art would have wanted to apply one of the recited methods to the Schmitt.

iii. *Claim 9 is not unpatentable*

Claim 9 further requires the mapping step to "us[es] information about a length of each of the sequence reads."

Schmitt does not disclose using information about length of each of the sequence reads. Instead, the petition materials merely assert that "[a] POSA would have understood that aligning (*i.e.*, mapping) reads with the Burrows-Wheeler aligner uses information about a length of each of the sequence reads." Pet. 64 (citing EX1077 at 1757; EX1002, ¶173). The petition does not explain what aspect of the BWA algorithm that allegedly provides this teaching.

iv. *Claim 12 is not unpatentable*

Claim 12 further requires that "the population of polynucleotides is tagged with n different unique identifiers, wherein n is no more than 100*z, wherein z is a mean of an expected number of duplicate molecules having the same start and stop positions in the sample."

None of the cited references disclose the expected number of duplicate molecules in a sample much less a tagging scheme based on such a number. As discussed above, the expected number of duplicates in a given amount of cfDNA was empirically determined by the inventor of the '822 patent. *See* Section V. Likewise, a tagging scheme based on the number of duplicates in a sample is novel to the '822 patent.

Petitioner's argument for claim 12 represents nothing beyond hindsight. Petitioner even acknowledges its reliance the empirical data in the '822 patent.

> The specification of the '822 patent defines "z" as a "measure of central tendency (e.g., mean, median, mode) of an expected number of duplicate molecules having the same start and stop positions" and states that "[i]t has been empirically determined that in a sample of about 10,000 haploid genome equivalents of human DNA, there are about 3 duplicate polynucleotides beginning at any given position." EX1001, 40:24-27; 41:10-13; EX1002, ¶178.

Pet. 66. Using the '822 patent's disclosure, Petitioner then calculates the expected number of duplicates in a 20 ng sample:

> Fan and Forshew disclose cfDNA amounts between 1-20 ng. EX1048, 16270, Table S1; EX1004, Table S6. A sample of 20 ng of DNA contains about 6,060 haploid genome equivalents, which would have approximately 1-2 duplicate polynucleotides beginning at any given position.

Pet. 66. No explanation is given for selecting a 20 ng sample size from the 1-20 ng range, as this too appears hindsight-based. Had Petitioner selected 1 ng, for example, there would have been no duplicate polynucleotides.

v. *Claim 13 is not unpatentable*

Claim 13 further requires that the bodily sample comprise "no more than 100 nanograms of polynucleotides."

Schmitt discloses using 3 µg of human colonic mucosa DNA. EX1011, 19:18-19, 19:58. That is, Schmitt uses samples that contain more than 300-fold more cellular DNA that the maximum amount of cfDNA required by claim 13.

The petition materials do not establish that a person of ordinary skill in the art would have had a reasonable expectation of success using a minute quantity of cfDNA.

VIII. OBJECTIVE INDICIA CONFIRM THE PATENTABILITY OF THE CHALLENGED CLAIMS

Petitioner's attack against the challenged claims can be dismissed solely based on the unaddressed limitations and host of other deficiencies detailed above. *See* Section VII. Additionally, objective indicia confirm the patentability of the claimed subject matter. *Kinetic Concepts, Inc. v. Smith & Nephew, Inc.*, 688 F.3d 1342, 1370-71 (Fed. Cir. 2012) (objective indicia demonstrate how a patented device is viewed in the marketplace and provide an unbiased indication regarding patentability); *Mintz v. Dietz & Watson, Inc.*, 679 F.3d 1372, 1378 (Fed. Cir. 2012) (objective indicia constitute independent evidence of nonobviousness) (citations omitted).

Here, ample evidence, including the FDA's designation of breakthrough status to the Guardant360 genomic test, demonstrate long-felt need and resulting commercial success of the claimed invention. It is well-settled that "there is a ***presumption of nexus*** for objective considerations when the patentee shows that the asserted objective evidence is tied to a specific product and that product 'is the

invention disclosed and claimed in the patent.'" *WBIP, LLC v. Kohler Co.*, 829 F.3d 1317, 1329 (Fed. Cir. 2016) (emphasis added). *See also* EX2025, ¶¶156-166.

Guardant360 is a "liquid biopsy" assay that provides clinically actionable sequence information from cfDNA molecules obtained from a routine blood draw from cancer patients. EX2034 at 4. The assay sequences a panel of genes commonly mutated in cancer and detects genetic aberrations such as single nucleotide variants (SNVs), indels, gene fusions, and copy number variants (CNVs). EX2029 at 5 (Figure 1). Guardant360 provides highly sensitive and accurate detection of genetic aberrations using a non-unique barcoding scheme in concert with bioinformatic algorithms specific for SNVs, indels, gene fusions, and CNVs. *Id.*; *see also* EX2030 at 3542-3544.

As Dr. Quackenbush explains, the Guardant360 test embodies at least claim 1 of the '822 patent. Guardant360 converts cfDNA obtained from a patient into non-uniquely tagged parent polynucleotides which are then amplified and sequenced. EX2029 at 5, 17-18; EX2030 at 3539, 3540, 3542, 3544. The resulting reads are then mapped to a reference genome and grouped into families based on start and stop position and identifier sequence. EX2029 at 19; EX2030 at 3540-3541. Finally, at each genetic locus sequence reads are collapsed to generate a base call and the frequency of one or more bases at each locus is determined. EX2029 at 19; EX2030 at 3544.

A. Long-Felt Need

Effective treatment of various cancers is often predicated on early detection of specific genomic alternations and accurate characterization of corresponding tumors. Numerous obstacles, however, have long stood in the way of reliably meeting these objectives. Traditional tumor-based genotyping assays are limited in the number of genes interrogated, require invasive biopsies, and often take upwards of 15 days before results are generated. EX2031, 1-2; *see also* EX2032, 1. Using cell-free DNA to detect early stage cancers is also fraught with unique challenges. EX2033, 16. Early attempts to use cfDNA lacked the necessary sensitivity and specificity for diagnosing and characterizing cancers. EX2033, 16 (describing limits in clinical applications and lack of genomic sensitivity). *See also* EX2025, ¶¶160-162.

Precision oncology "focuse[s] on matching cancer patients to personalized treatments based on the underlying molecular profile of their tumors." EX2034, 1. The industry has long faced "a critical need to expand the scope of precision oncology to enable precise detection, monitoring and selection of the appropriate intervention as early in the disease state as possible." EX2034, 1. That is, "[d]espite improvements to clinical outcomes and oncology drug development … precision oncology has not significantly impacted earlier stage cancer care." EX2034, 6. "Many early stage cancer patients receive only non-targeted

chemotherapy post-surgical resection of the tumor in the adjuvant setting and ad-hoc, symptomatic monitoring for recurrence. For early detection of cancer … the current standard of care is comprised of single protein biomarker tests or radiographic imaging, which can have challenges with high false positive rates." EX2034, 6. These long-standing hurdles were ones that Guardant, through the innovation of the '822 patent, effectively addressed. EX2025, ¶¶160-162

In January 2018, the Guardant360 test was granted Breakthrough Devices designation by the FDA. EX2034, 27; *see also* EX2036, 1. The Breakthrough Device designation is a prioritized review of products that "provide for more effective treatment or diagnosis of life-threatening or irreversibly debilitating diseases or conditions." EX2035, 1. Further, the designation is a recognition that the product "***demonstrates the potential to address unmet medical needs*** for such a disease or condition." 21 U.S.C. § 356(b)(1) (emphasis added). The Federal Circuit has confirmed that such expedited review is evidence of long-felt need. *Ferring BV v. Watson Labs. Inc.,* 764 F.3d 1401 (Fed. Cir. 2014) ("there was a long-felt and unmet need for a treatment … as the FDA recognized in granting 'fast track' status under 21 U.S.C. § 356(b)(1)"). EX2025, ¶¶160-162

Petitioner has previously asserted that such breakthrough designation is evidence of long-felt but unmet need. *Guardant Health, Inc., v. Foundation Medicine, Inc.,* IPR2017-01170, Paper 26 at 55-59 (arguing that FDA's

breakthrough designation demonstrates long-felt need and strongly supports non-obviousness). EX2025, ¶¶160-162

B. Commercial Success

The claimed invention, as embodied by Guardnt360, has seen widespread adoption in the industry. "Since its introduction in 2014, the Guardant360 assay has been ordered more than 70,000 times to help inform which therapy may be effective for advanced-stage cancer patients with solid tumors. More than 80 peer-reviewed studies have been published addressing its analytical validity, clinical validity, and clinical utility in multiple tumor types." EX2036, 2; *see also* EX2034, 13 (Guardant360 has been used "by more than 6,000 oncologists across dozens of cancer types, by more than 50 biopharmaceutical companies and by all 28 NCCN centers."). EX2025, ¶¶163-166.

The prevalence of Guardant360 has made it one of the "two most common commercially used liquid biopsies" EX2037, 988. Notably, the other most common liquid biopsy test is FoundationACT – Petitioner's infringing product. EX2037, 988. Indeed, the FoundationACT's infringement of the '822 patent is currently the subject of litigation in district court. EX2006. That is, the innovation of the '822 patent has market dominance, accounting for all the most common liquid biopsy methods available. EX2037, 988; EX2025, ¶¶163-166.

Reflecting the innovation of the '822 patent, Guardant360 has resulted in tremendous commercial success. *See* EX2032, 2-3 (attributing Guardant's financial growth and success to its genomic tests, including Guardant360). Sales of Guardant360 "accounted for primarily all of [Guardant's] revenue for the years ended December 31, 2016 and 2017, respectively, and a majority of [Guardant's] revenue for the year ended December 31, 2018." EX2034, 33. Guardant360 is so successful that the test is expected to "continue to account for the substantial majority of [Guardant's] revenue going forward." EX2034, 33. In 2019, Guardant's "[r]evenue was $60.8 million, a 181% increase from a year ago". Guardant's revenue also experienced a "year-over-year increase of more than 125%." EX2032, 2. Guardant also had "total revenues of $54.0 million for the quarter ended June 30, compared to $19.4 million in the same period of 2018, handily beating the $36.4 million that Wall Street had predicted on average." EX2038, 1; EX2025, ¶¶163-166.

The commercial success of Guardant360 is also evidenced by the growth in Guardant's stock price. Specifically, Guardant's "stock price has doubled [in 2019] and has nearly tripled since the company started trading publicly on Oct. 4, 2018." EX2032, 1; EX2025, ¶¶163-166.

IX. CONCLUSION

For at least the reasons set forth above, petitioner has failed to meet its burden and the challenged claims should be found *not unpatentable*.

Respectfully submitted,

Dated: December 4, 2019

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
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CERTIFICATE OF COMPLIANCE

Pursuant to § 42.24(d), the undersigned certifies that this paper contains no more than 14,000 words, not including the portions of the paper exempted by § 42.24(b). According to the word-processing system used to prepare this paper, the paper contains 13,204 words.

Respectfully,

Dated: December 4, 2019

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182

X. APPENDIX – LIST OF EXHIBITS

Exhibit No.	Description
2001	Guardant Health, Inc. v. Foundation Medicine, Inc., Case No. 17-cv-1616, Document 56, REDACTED Plaintiff's Opening Claim Construction Brief
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2024	Curriculum Vitae of Jay Shendure, M.D., Ph.D.
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CERTIFICATE OF SERVICE

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

FOUNDATION MEDICINE, INC.,
Petitioner,

v.

GUARDANT HEALTH, INC.,
Patent Owner.

Case No. IPR2019-00652
U.S. Patent No. 9,834,822

PETITIONER'S REPLY TO PATENT OWNER RESPONSE

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I. INTRODUCTION

Patent Owner's Response ("POR") confirms that the challenged claims of the '822 patent are unpatentable. Indeed, Patent Owner does not (and cannot) dispute that nearly all claim limitations are taught by the prior art under the Board's claim construction. Consequently, Patent Owner tries to avoid the prior art by offering new but improperly narrow interpretations. However, even under Patent Owner's construction, the prior art renders the claims unpatentable.

First, Patent Owner seeks to re-write the limitations of its claims in the guise of claim construction. But its proffered construction contradicts its own arguments in co-pending litigation, the deposition testimony of Patent Owner's own expert, Dr. Quackenbush, and the specification's teaching.

Second, Patent Owner's insistence that the prior art method is incompatible with cfDNA focuses on features that are either irrelevant to cfDNA (*e.g.*, shearing), mischaracterized by Patent Owner (*e.g.*, optimal fragment size), or not recited in the claimed method (*e.g.*, efficiency). Focusing on the relevant features clearly demonstrates that a person of ordinary skill in the art ("POSA") would have had a reasonable expectation of success in practicing the obvious method of the challenged claims.

Finally, Patent Owner's supposed objective evidence lacks any nexus to the '822 patent claims. It does not outweigh the compelling prima facie case of obviousness.

II. PRELIMINARY MATTERS

Dr. Shendure's declaration and testimony deserve little weight. First, he ***never reviewed*** the '822 patent specification or claims, and limited his review to EX1011, the Schmitt patent. EX1102, 53:5-54:4, 115:17-116:13. Second, it is unclear whether parts of his declaration are his own opinion. Despite neither reviewing Dr. Quackenbush's declaration nor conferring with him, Dr. Shendure concedes that significant portions of his declaration substantially or completely overlap with Dr. Quackenbush's. *Id.*, 31:9-39:12; *see also* EX2023, ¶¶19, 23-24, 28, 35; EX2025, ¶¶128-130, 132, 136. Third, Patent Owner's acquisition of Dr. Shendure's company, Bellwether Bio, in April 2019 (EX2023, ¶3) created an ongoing relationship. He will receive additional consideration if commercialization milestones are met, and currently receives compensation as Scientific Consultant to Patent Owner. EX1102, 16:16-18:20, 21:11-15; EX1099.

Lastly, although Drs. Shendure and Quackenbush both contend that Petitioner's POSA definition describes a person with "extraordinary" skill, they

offer no substantiation or alternative definition. EX2023, ¶15; EX1102, 46:3-49:20; EX2025, ¶23; EX1103, 13:16-15:3.

III. CLAIM CONSTRUCTION

The District Court has already construed the key claim terms of the '822 Patent. EX1108, 13-18. Yet, confronted with the Board's Institution Decision (Paper 12, "D.I."), Patent Owner now urges the Board to narrow the construction of "non-uniquely tagged," despite (1) initially urging the Board to construe the terms consistent with Patent Owner's previous district court constructions, Paper 7, n. 9 (citing EX2001), and (2) arguing for a broader construction in parallel litigation—even though the same standard applies. *See Phillips v. AWH Corp.*, 415 F.3d 1303, 1312-13 (Fed. Cir. 2005) (*en banc*). The Board should reject Patent Owner's arguments and reaffirm its construction, which is identical to the District Court's. *See Changes to the Claim Construction Standard for Interpreting Claims in Trial Proceedings Before the Patent Trial and Appeal Board*, 83 Fed. Reg. 51340, 51355 (Oct. 11, 2018) (prior district court construction is not binding but nevertheless informative).

A. Patent Owner Improperly Limits Claims to a Single Embodiment

Patent Owner now suggests that the '822 patent provides an express definition of "non-uniquely tagged," arguing that the term should be construed to mean "the

number of different identifiers can be at least 2 and fewer than the number of polynucleotides that map to the mappable base position." POR, 15-17 (citing EX1001, 41:42-46); EX2025, ¶¶61-64. But Patent Owner's argument ignores a fundamental claim construction tenet: claims should not be limited to a particular embodiment, even if it is the only embodiment. *See Phillips*, 415 F.3d at 1323-24.

The Board's construction tracks the express definition provided in the specification:

> A set of polynucleotides in the composition ***that map to a mappable base position*** in a genome can be non-uniquely tagged, that is, the number of different identifiers can be at least at least 2 and fewer than the number of polynucleotides ***that map to the mappable base position***.

EX1001, 41:42-46; D.I., 10.[1] The passage provides an express definition of "non-uniquely tagged" in one context—polynucleotides "***that map to a mappable base position***"—but the definition should not be limited to the embodiment being discussed. *See Edwards Lifesciences LLC v. Cook Inc.*, 582 F.3d 1322, 1334 (Fed. Cir. 2009) (affirming district court finding that "the specification's use of 'i.e.' signals an intent to define the word to which it refers … and that definition was not

[1] Unless otherwise noted, all emphasis is added.

limited to the embodiment being discussed."). Patent Owner fails to point to any language in the specification or prosecution history suggesting that the express definition is limited to polynucleotides that map to a mappable base position.

To the contrary, as Patent Owner argued in parallel litigation, the '822 patent discloses various embodiments of non-unique tagging. EX2001, 8. One is based on the number of duplicate molecules having the same start and stop positions. *Id.*; EX2025, ¶¶58-59. Others are based on a population of extracellular polynucleotides or polynucleotides that map to a reference position. *See, e.g.*, EX1001, 3:13-15, 18:39-42. The specification thus makes clear that "non-uniquely tagged" refers to various polynucleotide populations and is not limited to the subpopulation of polynucleotides that map to a mappable base position. "Even when the specification describes only a single embodiment, the claims of the patent will not be read restrictively unless the patentee has demonstrated a clear intention to limit the claim scope using 'words or expressions of manifest exclusion or restriction.'" *Liebel-Flarsheim Co. v. Medrad, Inc.*, 358 F.3d 898, 906 (Fed. Cir. 2004). Patent Owner's attempt to limit the claims to a single embodiment should be rejected.

B. Patent Owner's Claim Construction Is Inconsistent with Its District Court Arguments

In co-pending litigation, Patent Owner argued that the express definition at 41:42-46 in the '822 patent "clarifies that the total number of barcodes must plainly be less than the ***total number of polynucleotides***" but that it should nevertheless be disregarded when viewed in the full context provided in the specification. EX2001, 9. Patent Owner made no reference to the population of polynucleotides that map to a mappable base position, and simply argued that "the specification makes clear that the number of barcodes used in the 'non-unique' embodiments is far less than the billions of unique polynucleotides in the sample." *Id.*; *see also* EX1107.[2]

Now, Patent Owner newly submits that non-unique tagging is based on a subpopulation of "polynucleotides that map to the mappable base position." POR, 17. Under Patent Owner's construction, non-unique tagging requires the number of different identifiers to be at least two but fewer than the number of haploid

[2] During prosecution of related application 14/712,754, Patent Owner argued that 10ng of cfDNA "certainly contains billions of molecules" such that "using 'barcodes having from 5 to 1000 distinct barcode sequences' will necessarily tag the molecules non-uniquely." EX1107, 8.

equivalents of DNA in a sample. *See id.*, 18-19; EX2025, ¶¶67-69. As discussed below, this narrow claim reading is not only ambiguous, it is inconsistent with Patent Owner's district court arguments and Dr. Quackenbush's declaration and deposition testimony.

As Dr. Quackenbush admitted, and Patent Owner argued in co-pending litigation, non-unique tagging is demonstrated by a specific embodiment of the specification that describes "tag[ging] with n different unique identifiers, wherein n is at least 2 and no more than 100,000*z." *See* EX1103, 35:20-40:21 (admitting that this embodiment "specifically refers to the number of tags used in the claimed method"); EX2025, ¶58 (citing EX1001, 41:6-13); EX2001, 8 (citing EX1109, 41:15-17). According to the '822 patent, "z" is "an expected number of duplicate molecules having the same start and stop positions" and "in a sample of about 10,000 haploid genome equivalents of human DNA, there are about 3 duplicate polynucleotides beginning at any given position." EX1001, 40:24-27, 41:10-13. Thus, for a sample comprising 10,000 haploid copies of fragmented DNA, z=3, and the '822 patent teaches that the sample can be non-uniquely tagged with up to 100,000*3 (*i.e.*, 300,000) identifiers. *See id.*, 41:6-13. However, under Patent Owner's construction, a sample comprising 10,000 haploid copies cannot have more

than 10,000 fragments that map to a mappable base position and thus can only be non-uniquely tagged if there are fewer than 10,000 identifiers. *See* POR, 17-19, 25-26; EX2025, ¶¶67-69, 75-76. Patent Owner's proposed construction for non-unique tagging thus cannot be reconciled with the construction it advocated in district court, the teachings of the patent, or its own expert's testimony. In fact, Patent Owner's construction excludes at least this embodiment of non-unique tagging (*i.e.*, tagging with 100,000*z identifiers) and should therefore be rejected: "there is a strong presumption against a claim construction that excludes a disclosed embodiment." *In re Katz Interactive Call Processing Patent Litig.*, 639 F.3d 1303, 1324 (Fed. Cir. 2011).

Moreover, Patent Owner's construction introduces ambiguity and adds no clarity to the meaning of "non-uniquely tagged." *See Int'l Bus. Machines Corp. v. Intellectual Ventures II LLC.*, IPR2014-00660, Paper 58, 12 (PTAB Oct. 19, 2015) (rejecting construction that "adds no clarity"). Patent Owner's construction requires determining the number of polynucleotides that map to a "mappable base position." But a POSA would understand that a "mappable base position" may encompass multiple nucleotides, as one nucleotide cannot identify a genomic position. Rather, a sequence may be mappable when it is of sufficient length and complexity.

Moreover, the '822 specification explains that "the mappable position in the reference sequence is the ***locus of a tumor marker***..." (EX1001, 19:58-62) and provides "a method of estimating frequency of a base or ***sequence of bases at a locus***..." (EX1001, 29:46-49). A tumor marker can encompass thousands of nucleotides. *See, e.g.*, EX1004, 3. Thus, a POSA reading the specification would understand that "mappable base position" includes one or more nucleotides. *See also* EX1104, ¶¶11-13.

Furthermore, as Dr. Gabriel explained during cross-examination, for a single genomic copy fragmented into 200bp fragments, ***at least one fragment*** would map to a given mappable base position. EX2022, 53:9-55:11. However, sequence similarities may allow for confident mapping of ***one or more fragments*** to a given mappable base position because "[m]ost genomes contain at least some sequence that is repetitive or close to repetitive on the length scale of the reads." EX1083, 1851; *see also* EX2022, 53:9-55:11. Additionally, some CNVs can add copies of genes or gene regions such that multiple polynucleotide fragments per haploid copy would map to a mappable base position located within the span of such a region. EX1104, ¶14. Thus, Patent Owner's construction creates further ambiguity as to the upper limit of tags that allow for "non-unique tagging." It should be rejected.

For these reasons, Patent Owner's contradictory claim construction arguments should be rejected.

IV. THE COMBINATION OF PRIOR ART DISCLOSES ALL LIMITATIONS OF THE CHALLENGED CLAIMS

Even if the Board accepted Patent Owner's erroneous construction of "non-unique tagging,"[3] *supra* Section III, the prior art would still disclose the limitations and render the claims obvious.

A. Claim 1 Is Unpatentable

First, Petitioner has demonstrated Schmitt teaches non-uniquely tagged parent polynucleotides when calculating the number of unique tags relative to the number of parent polynucleotides. Pet., 42-46. According to Patent Owner, a sample of one genome equivalent has "far fewer fragments than 'millions of fragments' and certainly less than tags generated by a 4-mer." POR, 29; EX2025,

[3] Patent Owner also contends that the combination of Schmitt, Fan, and/or Forshew does not render claim 1 obvious. *See* POR, 33-35; EX2025, ¶¶94-101. But Patent Owner argues against the references individually, which cannot show non-obviousness where the rejections are based on combinations of references. *See In re Merck & Co., Inc.*, 800 F.2d 1091, 1097 (Fed. Cir. 1986).

¶85. Patent Owner contends that Schmitt requires capturing fragmented DNA that targets a 300-20,000bp region, which would result in no more than one hundred 200bp fragments per genome equivalent. POR, 29. Patent Owner is incorrect because Schmitt discloses tagging DNA fragments ***before*** target capture, not after. *See* EX1011, Fig. 1, 20:16-30. One genome equivalent fragmented into 200-500bp ***would*** comprise millions of fragments. Under the Board's construction, tagging this sample with a 4-mer (*i.e.*, 65,536 different tag combinations) as disclosed by Schmitt, would produce non-uniquely tagged parent polynucleotides because "the number of different identifiers attached to the polynucleotides is at least 2 and fewer than the number of polynucleotides." D.I., 9; Pet., 44-45; EX1104, ¶¶17-18.

A POSA would further understand that a DNA sample for NGS would comprise significantly more than one genome equivalent.[4] EX1104, ¶19. As Petitioner explained, Fan and Forshew disclose sequencing cfDNA amounts between 1-20ng. Pet., 41-42. A sample of 20ng of DNA contains about 6,060

[4] Schmitt teaches tagging 750ng of DNA followed by amplifying 375ng of DNA. EX1011, 20:9-22.

haploid genome equivalents, or billions of fragments,[5] and would also be non-uniquely tagged with a 4-mer under the Board's construction. Pet., 66. Additionally, Kinde 2012 (EX1059) and Liao (EX1052) disclose sequencing experiments starting with 33ng and 5-30ng cfDNA, respectively. EX1059, 2; EX1052, 94.

Second, even under Patent Owner's construction, the cited references teach "non-uniquely tagged parent polynucleotides." Petitioner explained that the cited art discloses tagging a population of cfDNA with n different unique identifiers, wherein n is no more than 100*z. Pet., 66-67. Specifically, a sample comprising 20ng cfDNA, or ~6,060 genome equivalents, is non-uniquely tagged with a 1-mer tag that yields 16 different tag combinations. *Id.* Under Patent Owner's construction, this sample is non-uniquely tagged because there are fewer tags than genome equivalents. *See* POR, 26; EX2025, ¶76. The same would be true for a 2-mer tag, which yields 256 different tag combinations. *See* Pet., 70; EX1104, ¶20.

Moreover, Petitioner's explanation of non-uniquely tagging DNA using a 4-mer is not unreasonable. EX1104, ¶21. Patent Owner argues that "65,537 haploid

[5] One genome equivalent of cfDNA comprises ~20,000,000 polynucleotide fragments. *See* EX1001, 40:15-18.

copies" of the genome "would require an extraordinary amount of sequencing." POR, 26; EX2025, ¶78. But Petitioner never contended that the entire population of parent polynucleotides must be sequenced nor, as Patent Owner acknowledges, does Schmitt require it. POR, 26; EX2025, ¶79. If anything, under Patent Owner's construction, the scope of claim 1 of the '822 patent encompasses non-uniquely tagging significant amounts of genome equivalents. Claim 11 recites tagging "with from 10 to 100,000 different identifiers." EX1001, 63:11-13. Under Patent Owner's construction, non-uniquely tagging with 100,000 identifiers requires a sample to have more than 100,000 polynucleotides that map to a mappable base position, *i.e.*, more than 100,000 haploid equivalents. Because claim 11 depends from claim 1, claim 1 must encompass non-uniquely tagging with a range of less than 10 but more than 100,000 identifiers. *See Pfizer, Inc. v. Ranbaxy Labs. Ltd.*, 457 F.3d 1284, 1291-92 (Fed. Cir. 2006). Thus, claim 1 encompasses non-uniquely tagging from less than 10 to more than 100,000 haploid equivalents.

Third, Patent Owner fails to distinguish Schmitt's hybrid method from the approach of claim 1 of the '822 patent. Contrary to Patent Owner's assertions (POR, 27-31), Schmitt is not limited to unique tagging. *See* Pet., 43-46; EX1002, ¶¶126-127. Schmitt discloses a "hybrid method" of tagging parent polynucleotides using

"a combination of sheared ends and a shorter n-mer tag." EX1002, ¶126 (citing EX1011, 9:1-13); *see also* EX1011, 37:22-31 (using single molecule identifier ("SMI") tags "that alone or in combination with the target nucleic acid shear points uniquely labels" DNA fragments). Patent Owner's attempt to distinguish claim 1 by relying on a narrow reading of non-unique tagging (*see* POR, 28, 32) is fundamentally flawed. *Supra* Section III.

Patent Owner also relies on the shearing of cellular DNA to distinguish claim 1 from Schmitt. POR, 31-33; EX2025, ¶¶92-93. However, a POSA trying to apply the hybrid method of Schmitt to cfDNA would not have sheared the cfDNA because the POSA would have understood that utilizing cell-free fragments of ~140-170bp is functionally equivalent to using slightly larger fragments of genomic DNA as in Schmitt. *See* Pet., 39-40 (citing EX1002, ¶118); EX1052, 94 ("plasma DNA molecules were already fragmented in nature, so no additional fragmentation step was required"). Even Patent Owner's expert Dr. Shendure concedes that researchers working with cfDNA in 2012 would not have sheared the DNA. EX1102, 119:16-121:14 (admitting that most groups working with cfDNA in 2012 "did not shear the material."). Shearing is not required to apply the hybrid method to cfDNA because

a POSA would understand that the start and stop positions of cfDNA encompass the sequences at the sheared ends. Pet., 51; EX1002, ¶143. *See also* EX1104, ¶¶22-25.

B. Dependent Claims 2, 5, 9, 12 and 13 Are Unpatentable

1. Claim 2 Is Unpatentable

Patent Owner contends that the Petition "fail[s] to point to any disclosure of detection of CNVs" (POR, 53) but ignores Petitioner's explanation that SMI tags allow for "single-molecule counting," which "is applicable for 'accurate detection of ***altered genomic copy number*** … ***such as trisomy 21***.'" Pet., 58 (citing EX1011, 18:3-21; EX1002, ¶¶158-159).

Patent Owner also contends that the Petition fails to explain the "different types of mutations that could be identified from the same data set." POR, 54 (citing Pet., 58). But Petitioner identified "single nucleotide substitutions, indel, gene fusion" and Dr. Gabriel illustrated these mutations. Pet., 58-59 (citing EX1002, ¶¶160-161, ¶37 (figure); EX1005, Figure 3; EX1067; EX1068). *See also* EX1104, ¶¶26-27.

2. Claim 5 Is Unpatentable

It would have been obvious to a POSA to collapse sequence reads in each family to yield a consensus sequence by the claimed method (Pet., 60; EX1002, ¶167) based on the common knowledge of using a known base calling method, *e.g.*,

voting, to generate a consensus sequence. EX1002, ¶167; *see also KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 418 (2007).

3. Claim 9 Is Unpatentable

A POSA would have understood that aligning (*i.e.*, mapping) reads with the Burrows-Wheeler aligner uses information about a length of each of the sequence reads. Pet., 64 (citing EX1077, 1757; EX1002, ¶173). According to Li and Durbin (EX1077), "[d]etermining the allowed maximum number of differences" is based on "a read of length *m*." EX1077, 1757; *see also* EX1104, ¶29.

4. Claim 12 Is Unpatentable

Claim 12 is directed to a number of identifiers for non-unique tagging, *i.e.*, no more than 100*z. The combination of Schmitt, Fan, and Forshew disclose tagging a population of 20ng cfDNA with no more than 100*z identifiers. Pet., 66-67; EX1002, ¶¶178-181.

5. Claim 13 Is Unpatentable

Patent Owner disputes the obviousness of claim 13 based solely on the disclosure of Schmitt. POR, 56-57. But one cannot show non-obviousness by attacking references individually where the rejections are based on combinations of references. *See Merck*, 800 F.2d at 1097.

V. A POSA WOULD HAVE BEEN MOTIVATED TO COMBINE THE PRIOR ART

Patent Owner contends that a POSA would not understand Schmitt's reference to fetal aneuploidy as "contemplating application of DCS to fetal aneuploidy detection" because it is only part of the "introductory remarks." POR, 35 (citing EX2025, ¶¶102-110; EX2023, ¶20). But Schmitt's remarks are not merely introductory.

As the Board recognized, Schmitt discloses that "deep sequencing" has been used "in a variety of fields," including "prenatal screening for fetal aneuploidy" and "early detection of cancer." D.I., 25; *see also* Pet., 34. Schmitt explains that Duplex Consensus Sequencing ("DCS") overcomes limitations in sequencing accuracy, including those in deep sequencing of ***heterogeneous mixtures***. Pet., 35 (citing EX1011, 2:56-62, Abstract; EX1002, ¶105; EX1047, Abstract). By 2012, barcode-based sequencing approaches had been predicted and successfully used to detect aberrations (*e.g.*, fetal aneuploidy) in cfDNA. *Id.*, 38 (citing EX1058; EX1059; EX1002, ¶113). In fact, Schmitt states that SMI tags allow for "single-molecule counting for accurate determination of DNA or RNA copy number" that is applicable for "accurate detection of altered genomic copy number … such as trisomy 21." EX1002, ¶158 (internal citation omitted); Pet., 58 (citing EX1011,

18:3-21; EX1002, ¶¶158-159). While Patent Owner contends that "there would be no good reason to apply Schmitt's DCS method to detecting fetal aneuploidy" (POR, 35), Schmitt clearly provides one. EX1104, ¶¶33-36. *See also* EX1102, 68:13-69:3, 70:14-71:12.

Patent Owner also argues that Schmitt and Forshew are at most analogous art and that their use of NGS "is not evidence that a POSA would have wanted to … combine[] these methods." POR, 37-38 (citing EX2025, ¶¶108-110). Dr. Quackenbush admits, however, that both Schmitt and Forshew use error correction methods to distinguish errors from mutations: Forshew relies on redundant sequencing and Schmitt uses molecular barcoding. *See* EX2025, ¶¶31-33. Thus, Schmitt and Forshew do not merely use NGS, they each apply error correction methods to correctly identify mutations. *See* Pet., 37 (citing EX1002, ¶109); EX1104, ¶38. As the Board stated, "Schmitt's DCS method was a reasonable substitute for the deep sequencing techniques used in the prior art—such as those techniques Fan and Forshew used…" D.I., 25-26. *See also* EX1104, ¶¶37-38.

A POSA would have been motivated to combine the prior art to arrive at the claimed invention. EX1104, ¶39.

VI. A POSA WOULD HAVE HAD A REASONABLE EXPECTATION OF SUCCESS

The record demonstrates that a POSA would have had a reasonable expectation of success in practicing the obvious method of the challenged claims—evidence which Patent Owner mischaracterizes or ignores. Patent Owner also attacks the efficiency of the prior art method. But efficiency is not required to show reasonable expectation of success because, as the Board correctly noted, and as Patent Owner's expert admitted, claim 1 of the '822 patent does not recite conversion efficiency. *See* D.I., 26; EX1103, 92:18-93:9.

To the extent that efficiency is relevant to applying DCS to cfDNA, the '822 patent explains that efficiency is only one of two tools that may contribute to detection sensitivity, and is ***not required***. *See* EX1001, 32:33-53 ("The present disclosure further provides methods and systems for detecting with high ***sensitivity*** genetic variation in a sample…"); *see also id.* ("The methods involve using ***one or both*** of the following tools…"). Moreover, despite insisting that efficiency is relevant to applying DCS to cfDNA (*see* POR, 43-45, 49-50), Patent Owner contends that sensitivity "does not address the applicability of DCS to cfDNA." *Id.*, 39. In fact, DCS would have been "sensitive enough to detect rare mutations irrespective of the source of the sequenced DNA." Pet., 39 (citing EX1002, ¶115).

Patent Owner's SEC filing explains that sensitivity is one of "two key performance characteristics that are critical for a liquid biopsy test" (EX2034, 19/193) but "the sensitivity and specificity of conventional next-generation sequencing ... is inadequate for liquid biopsies due to the low concentrations of ctDNA in blood [*i.e.*, mutation frequencies of 0.46% to below 0.01%]." *Id.*, 12/193. Because DCS can detect mutant sequences with a sensitivity of 0.01% or better (*see* Pet., 38-39 (citing EX1011, 30:7-10; EX1002, ¶115)), the sensitivity of DCS would have been adequate for liquid biopsies. EX1104, ¶¶41-44. Thus, the sensitivity of DCS, which Patent Owner does not dispute, plainly addresses the applicability of DCS to cfDNA. *See* POR, 39. Patent Owner's arguments regarding efficiency are moot.[6]

Patent Owner further relies on Schmitt's shearing and size-selecting steps to contend that (1) Schmitt's tagging, amplifying, and sequencing steps would not operate similarly for cfDNA and (2) the 200-500bp DNA fragments of Schmitt are

[6] Patent Owner's arguments regarding references that allegedly demonstrate DCS "is inefficient and poorly suited for cfDNA" are also moot. *See* POR, 43-45; *see also* EX1104, ¶¶51-53.

not similar in size to cfDNA. POR, 41. Patent Owner contends that shearing is incompatible with cfDNA (*id.*, 50-51) and that the Petition concedes that shearing is "necessary" to make fragments "'compatible with amplification and NGS sequencing.'" *Id.*, 42 (citing EX1002, ¶118; Pet., 39-40). These arguments are flawed.

First, a POSA trying to apply the hybrid method of Schmitt to cfDNA ***would not have sheared cfDNA***. *Supra* Section IV.A; Pet., 39-40 (citing EX1002, ¶118). Moreover, Petitioner never "conceded" that shearing was necessary. Instead, Dr. Gabriel explained that "200-500bp fragments and naturally fragmented cfDNA are equally amenable to amplification and sequencing…" *See* EX1002, ¶118; *see also* EX1104, ¶¶45-47.

Second, Schmitt's disclosure of an "optimal range" does not limit DCS to 200-500bp fragments. *See* POR, 41, 46; EX2025, ¶¶118, 129-130; EX2023, ¶¶23-24. Rather, Schmitt discloses "size-selecting for ***appropriate length*** fragments." EX1011, 3:53-56. A POSA would have understood that 140-170bp fragments were an "appropriate length" for DCS because Schmitt discloses that "SMI-containing adaptors … may be substituted for standard sequencing adaptors. Thus, use of ***DCS does not require any significant deviations from the normal workflow of sample***

preparation for Illumina DNA sequencing." EX1011, 18:44-51. Fan discloses cfDNA sample preparation for the Solexa/Illumina platform "according to the manufacturer's protocol with slight modifications."[7] EX1048, 16270-71. Similar to DCS, Fan blunt-ended and ligated adaptors to DNA, followed by amplification and sequencing. *Id.* As Dr. Shendure admits, "there's nothing inherently incompatible with the broad class Illumina sequencers" and 140-170bp cfDNA fragments. EX1102, 130:5-11. Accordingly, a POSA would have understood that cfDNA fragments were an appropriate length for DCS. *See also* EX1104, ¶¶48-50.

Patent Owner also contends that "[e]ven if other methods using NGS had been applied to cfDNA," this does not establish that DCS is applicable to cfDNA. POR, 38. But Patent Owner fails to acknowledge that the "methods for barcoding" of EX1049 (POR, 38-39; EX2025, ¶112 (similar)) comprise a ***molecular barcoding*** error correction method analogous to DCS. *See* EX1002, ¶¶113 (citing EX1058; EX1059), 118 (citing EX1049; EX1059); *see also* EX1056 (explaining that DCS is

[7] As Dr. Quackenbush admits, DNA ligation kits were commercially available and "people may have substituted their own [sequencing] adapters." EX1103, 133:18-134:10.

analogous to the method of EX1049). Similarly, Patent Owner fails to acknowledge that the "method for detecting fetal aneuploidy by counting sequencing reads" of Kinde 2012 (POR, 39; EX2025, ¶112), comprised ***tagging the cfDNA with barcodes*** according to the method of Kinde 2011. *See* EX1002, ¶118 (citing EX1049; EX1059).

VII. DCS IS COMPATIBLE WITH CELL-FREE DNA

Patent Owner also argues that DCS is incompatible with cfDNA by mischaracterizing or ignoring the teachings of Schmitt. *See* POR, 45-53. But as Petitioner explained, a POSA would have understood that DCS is compatible with cfDNA because DCS is compatible with existing sequencing workflows and can "play a general role in next generation DNA sequencing." *See* Pet., 30 (citing EX1002, ¶99), 54-55 (citing EX1002, ¶¶151-152); *see also supra* IV.A.

Without citing any evidence, Patent Owner first falsely asserts that Dr. Gabriel conceded that "a POSA would have considered Schmitt incompatible with cfDNA analysis." POR, 19-20, 46. While Dr. Gabriel recognized that cfDNA analysis was "challenging and requires specialized methods," (POR, 7 (citing EX2003)), she ***also*** recognized that "error-correcting with molecular barcodes" could address this challenge. EX2003, 3. Patent Owner's expert, Dr. Quackenbush, does not dispute that "one might try [molecular barcoding methods] to solve the

problem of getting accurate mutation calls from cell-free DNA." EX1103, 140:13-142:16; *see also* EX1104, ¶¶54-56.

Patent Owner argues that "Schmitt requires large amounts of cellular DNA in its assays" but "it is simply not feasible to obtain cfDNA in microgram quantities from patients." POR, 49 (citing EX2023, ¶32). But Schmitt does not disclose any such "requirement" nor does Patent Owner cite to any such disclosure in Schmitt. A POSA would not have read Schmitt to exclude the smaller amounts of cfDNA typically available. Moreover, a POSA starting with cfDNA would not be required to start with the same amount as cellular DNA. *See* EX1057, 5 ("The amount of ***fragmented DNA*** actually used in the PCR amplification step is typically in ***the low-attomole range***, so the ***theoretical amount of starting DNA needed for [DCS] is extremely low.***"). Importantly, as discussed above, Schmitt discloses that DCS is compatible with existing sequencing workflows, *e.g.*, the Solexa/Illumina protocol used by Fan, which Fan applied to 1-8ng of cfDNA. EX1048, 16270-71. A POSA would understand and expect that DCS would operate in a similar manner whether one started with cellular or cfDNA. Pet., 39 (citing EX1002, ¶118). *See also* EX1111, 132:23-25 ("Whether or not polynucleotides are extracellular or not, the

sequencing machine doesn't care. It will generate sequence data."); EX1103, 66:15-67:6; EX1104, ¶¶57-58.

Patent Owner contends that Schmitt instructs steps that discard sample and data. POR, 47; EX2023, ¶25; EX2025, ¶131. Dr. Shendure concedes the contrary. *See* EX1102, 75:22-79:17 ("the actual manipulations are related to the removal of data rather than molecules"). Indeed, Schmitt teaches data processing steps that filter out reads that do not meet certain quality thresholds. *See, e.g.*, Pet., 64-65 (citing EX1002, ¶¶174-176), 69-70 (citing EX1002, ¶¶191-192). Contrary to Patent Owner's assertion that Schmitt's randomly generated SMI tags lead to data loss (POR, 47-48; EX2023, ¶¶26-29; *see also* EX2025, ¶¶132-133), Schmitt discloses SMIs can be ***known*** sequences. *See* EX1011, 7:23-27 ("The degenerate or semi-degenerate n-mer sequences may be generated by … preparing and annealing a library of individual oligonucleotides of known sequence"). *See also* EX1104, ¶59.

Patent Owner further contends that the "low diversity" of cfDNA[8] compared to sheared DNA results in data loss because Schmitt allegedly discards fragments with identical shear points. POR, 51-52; EX2023, ¶¶35-37. But Schmitt recognizes that sheared ends have limited diversity and teaches using SMI tags ***with*** the sheared ends to increase diversity. *See, e.g.*, EX1011, 9:6-9 ("there are a limited number of shear points flanking any given genomic position and thus the power to sequence deeply is increased via ***inclusion of the SMI tag***"), 26:67-27:8. In fact, Schmitt explains that using SMI tags "minimize[s] the loss of data by overcoming the intrinsic limitations of using shear points to identify PCR duplicates." *See id.*, 18:22-43. A POSA would have understood that even if naturally fragmented cfDNA is less diverse than sheared DNA, the use of n-mer tags according to Schmitt's hybrid method would provide sufficient diversity. *See also* EX1104, ¶¶60-65.

[8] It is unclear what Patent Owner means by "low diversity" given its assertion that 10,000 haploid equivalents of fragmented cfDNA have a diversity of "about 70 billion [] differently sequenced polynucleotide molecules." *See* POR, 12-13 (citing EX1001, 39:24-29).

VIII. PATENT OWNER'S ALLEGED OBJECTIVE EVIDENCE CANNOT ESTABLISH NON-OBVIOUSNESS

Patent Owner's assertions of objective indicia (POR, 57-62) cannot demonstrate non-obviousness.

A. No Presumption of Nexus

Patent Owner asserts a "presumption of nexus" by mapping prior art-disclosed claim features to Guardant360. POR, 57-58. But the claimed invention is not "coextensive" with Guardant360. *See Fox Factory, Inc. v. SRAM, LLC*, 944 F.3d 1366, 1373 (Fed. Cir. 2019). As the Federal Circuit recently explained, where a product "include[s] unclaimed features that the patentee describes as 'critical' to the product's ability" to perform, the product is not "coextensive" with the claim. *Id.*, 1374-75.

Here, Patent Owner and Dr. Quackenbush agree that the "two ***key performance characteristics***" of Guardant360 "that are critical" are "***sensitivity***, which refers to the level of ctDNA in circulation at which the technology reliably detects variants for a given input sample amount," and "***specificity***, which is the probability that a given test result is accurate." EX2034, 13; EX1103, 82:16-83:10, 85:3-87:11. *See also* POR, 59-60 (asserting "a critical need" for methods that had "the necessary sensitivity and specificity"). Yet as Dr. Quackenbush admitted,

neither of these purported "key" and "critical" features are claimed. EX1103, 91:22-94:11. To the extent Patent Owner contends that a nexus is established based on prior art-disclosed claim features in Guardant360, the Federal Circuit has long rejected such arguments.[9] *See Novartis AG. V. Torrent Pharm. Ltd.*, 853 F.3d 1316, 1331 (Fed. Cir. 2017). Thus, the '822 patent claims are not entitled to a presumption of nexus. *See Fox,* 944 F.3d at 1375; EX1104, ¶¶66-68.

B. Patent Owner's Evidence of Objective Indicia Is Insufficient

Patent Owner's evidence regarding long-felt need and commercial success are also inadequate. The only evidence of long-felt need that Patent Owner attempts to tie to Guardant360 is FDA's "Breakthrough" designation. *See* POR, 59-61. But, as explained above, Guardant360 is not co-extensive with the claims, and Patent Owner makes no attempt to tie FDA's designation to any claimed feature. *See id.*, 59-61; EX20205, ¶162; *Novartis*, 853 F.3d at 1330-31; EX1104, ¶¶66-68. Thus, without a

[9] The '822 specification does not purport to have invented non-unique tagging. *See* EX1001, 38:20-25.

nexus to the claimed invention, this evidence deserves little weight.[10]

Patent Owner's commercial success "evidence" is even more tenuous. For example, Patent Owner and Dr. Quackenbush rely on several documents that Dr. Quackenbush admitted address purported market penetration and financial success based on ***multiple*** Guardant products, only one of which—Guardant360—Dr. Quackenbush analyzed as allegedly embodying the claimed invention. *See* POR, 61-62; EX2025, ¶¶163-166; EX2034; EX2032[11]; EX1103, 73:18-82:11. Additionally, Dr. Quackenbush admitted that many factors affected whether Guardant360 was commercially adopted, including regulatory considerations, payer

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[10] In contrast, Petitioner's prior reliance on "breakthrough designation" directly linked the benefits recognized by FDA about the embodying product, FoundationOne CDx™, to the claimed features—"in particular, the novel use of multiple tailored bait sets, wherein the bait sets have unique preselected efficiencies for selection for their targets, to provide an accurate method for detecting numerous classes of somatic mutations in a single, comprehensive assay." *Guardant Health, Inc. v. Foundation Medicine, Inc.*, IPR2017-001170, Paper 26, 58.

[11] These documents are also unreliable on their face. *See, e.g.*, EX1103, 74:12-76:1.

coverage, reimbursement, and clinical utility—but none are covered by the '822 patent claims. EX1103, 72:5-73:17; EX2034, 3-4, 20. Dr. Quackenbush also admitted he is not an expert in the relevant fields, and thus his improper lay opinions regarding Patent Owner's revenues and stock prices should be ignored. *See* EX1103, 10:10-19; EX2025, ¶¶163-166.

Thus, Patent Owner's objective indicia evidence is unpersuasive and does not dislodge the determination that the claims are obvious. *See Leapfrog Enters. v. Fisher-Price, Inc.*, 485 F.3d 1157, 1162 (Fed. Cir. 2007); *In re Huai-Hung Kao*, 639 F.3d 1057, 1068 (Fed. Cir. 2011).

IX. CONCLUSION

Petitioner requests that the Board cancel the challenged claims.

Date: March 3, 2020

Respectfully submitted,

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CERTIFICATE OF COMPLIANCE

I hereby certify that the word count for the foregoing **PETITIONER'S REPLY TO PATENT OWNER RESPONSE** totals **5598** words, excluding the parts which are exempted by 37 C.F.R. § 42.24(a)(1).

CERTIFICATE OF SERVICE

The undersigned hereby certifies that the foregoing **PETITIONER'S REPLY TO PATENT OWNER RESPONSE** was served electronically in its entirety on March 3, 2020 via electronic mail to the following attorneys of record:

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RESEARCH ARTICLE

ERASE-Seq: Leveraging replicate measurements to enhance ultralow frequency variant detection in NGS data

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Citation: Kamps-Hughes N, McUsic A, Kurihara L, Harkins TT, Pal P, Ray C, et al. (2018) ERASE-Seq: Leveraging replicate measurements to enhance ultralow frequency variant detection in NGS data. PLoS ONE 13(4): e0195272. https://doi.org/10.1371/journal.pone.0195272

Editor: Yan W. Asmann, Mayo Clinic Minnesota, UNITED STATES

Received: September 7, 2017

Accepted: March 19, 2018

Published: April 9, 2018

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files. All sequencing data has been uploaded to the NCBI SRA database under BioProject Accession Number PRJNA389733. Additional details may be obtained by contacting the corresponding author.

Funding: The studies were funded by Fluxion Biosciences, Swift Biosciences and Illumina. No individual authors received specific funding for this work. The affiliated companies provided support in the form of salaries for authors NKH, CIZ (Fluxion

Abstract

The accurate detection of ultralow allele frequency variants in DNA samples is of interest in both research and medical settings, particularly in liquid biopsies where cancer mutational status is monitored from circulating DNA. Next-generation sequencing (NGS) technologies employing molecular barcoding have shown promise but significant sensitivity and specificity improvements are still needed to detect mutations in a majority of patients before the metastatic stage. To address this we present analytical validation data for ERASE-Seq (Elimination of Recurrent Artifacts and Stochastic Errors), a method for accurate and sensitive detection of ultralow frequency DNA variants in NGS data. ERASE-Seq differs from previous methods by creating a robust statistical framework to utilize technical replicates in conjunction with background error modeling, providing a 10 to 100-fold reduction in false positive rates compared to published molecular barcoding methods. ERASE-Seq was tested using spiked human DNA mixtures with clinically realistic DNA input quantities to detect SNVs and indels between 0.05% and 1% allele frequency, the range commonly found in liquid biopsy samples. Variants were detected with greater than 90% sensitivity and a false positive rate below 0.1 calls per 10,000 possible variants. The approach represents a significant performance improvement compared to molecular barcoding methods and does not require changing molecular reagents.

Introduction

Next-generation sequencing (NGS) has opened the door to personalized medicine by drastically reducing the time and cost required to assess an individual's nucleic acid composition[1]. This has allowed for the successful identification of germline mutations relevant to inherited genetic disorders[2], cancer predisposition[3], and drug sensitivity[4] among others. It has also provided a means of monitoring tumor material for somatic mutations, providing physicians with crucial information that can guide the course of treatment[5]. The massive read numbers produced by NGS technologies also provide the possibility to look with

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Biosciences), AM, LH, TH (Swift Biosciences), PP and CR (Illumina). The respective companies did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Competing interests: The authors are employed by Fluxion Biosciences, Swift Biosciences and Illumina. No individual authors received specific funding for this work. The affiliated companies provided support in the form of salaries for authors NKH, CIZ (Fluxion Biosciences), AM, LH, TH (Swift Biosciences), PP and CR (Illumina). The respective companies did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section. Authors of this paper declare that their employment did not affect their interpretation of the scientific data. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

unprecedented depth into a biological sample, identifying low frequency DNA variants buried at fractions of a percent relative to a wild-type background. One promising application is the liquid biopsy for cancer detection and monitoring which allows for the identification of clinically actionable somatic variants from blood samples, circumventing the need for expensive and invasive repeat tissue biopsies[6–8].

The ability to leverage the high sequence depth provided by NGS instruments to accurately identify ultralow frequency mutations has been technically problematic due to errors introduced by both library preparation and sequencing chemistries[9]. Common bioinformatics workflows utilize metrics such as base quality, read depth, allele frequency, and strand-bias to eliminate the most salient errors in NGS data[10, 11] allowing sensitive and specific variant identification down to 2–5% allele frequency (AF). This has provided a sound methodology for identifying somatic mutations from solid tumor biopsies[5]. However, the cell free DNA obtained in liquid biopsy samples often harbors somatic mutations in the 0.01–1% range[6–8, 12], requiring specialized molecular protocols and algorithms designed to systematically reduce error rates. Molecular barcoding has shown promise in drastically lowering the limit of detection for variants in NGS data. However, the proof of principle experiments demonstrating high accuracy at ultralow allele frequencies have required extremely high read depths and DNA inputs while demonstrating efficacy only over very small genomic regions[13, 14]. Practical attempts to apply this technology to larger clinically relevant sequence spaces have not attained the same performance[15, 16], showing loss of accuracy in the 0.5–2% AF range. A notable exception is the CAPP-Seq iDES method that combines barcoding with a background-aware caller and provides excellent sensitivity to below 0.1%, however high specificity is sacrificed and false positive counts are kept low by confining variant tests to a tiny fraction of the panel[17]. While several cfDNA based tests have been commercialized, significant questions remain as to their fidelity in detecting tumor biopsy variants and agreement across different tests is low [18–20], demonstrating the need for tests with increased sensitivity an ddecreased false positive rates.

In the work presented here we apply a new approach to ultralow frequency variant detection. ERASE-Seq combines the power of technical replication with background-aware variant calling (Fig 1) to achieve high-resolution DNA variant calls to below 0.1% allele frequency in highly multiplexed amplicon panels. The basis of our method is elimination of the two major categories of false positives in NGS low frequency variant data: recurrent artifacts and stochastic errors. Recurrent artifacts occur at error-prone loci that are predisposed to base misincorporation either during library prep or sequencing[21, 22]. ERASE-Seq utilizes a set of wild-type control DNA technical replicates that have undergone library prep and sequencing to quantify the error background for each variant across the multiplexed amplicon panel. Stochastic errors also occur during the library preparation and sequencing processes, especially below 0.5% allele frequency. These occur randomly throughout the sequencing space as a function of polymerase error rates[9, 13]. Consequently, they can only be eliminated using technical replication, and we observe decreasing false positive rates with increasing replicate number. ERASE-Seq brings these two error elimination strategies together by employing a statistical test between count values in sample and reference replicates for each observed variant (Fig 1). Recurrent artifacts are eliminated due to their presence in reference replicates, and stochastic errors by their inconsistent signal in sample replicates (Fig 1). We demonstrate the ability of ERASE-Seq to accurately detect low frequency SNVs and indels on three different highly multiplexed oncology amplicon panels: 56G (Swift Biosciences), TST15 (Illumina), and Spotlight 59 (Fluxion Biosciences). Our data show perfect sensitivity and specificity to 0.3% allele frequency and maintain high performance below 0.1%. We analyze the composition of false positive calls using standard methodologies in terms of recurrent versus stochastic errors and

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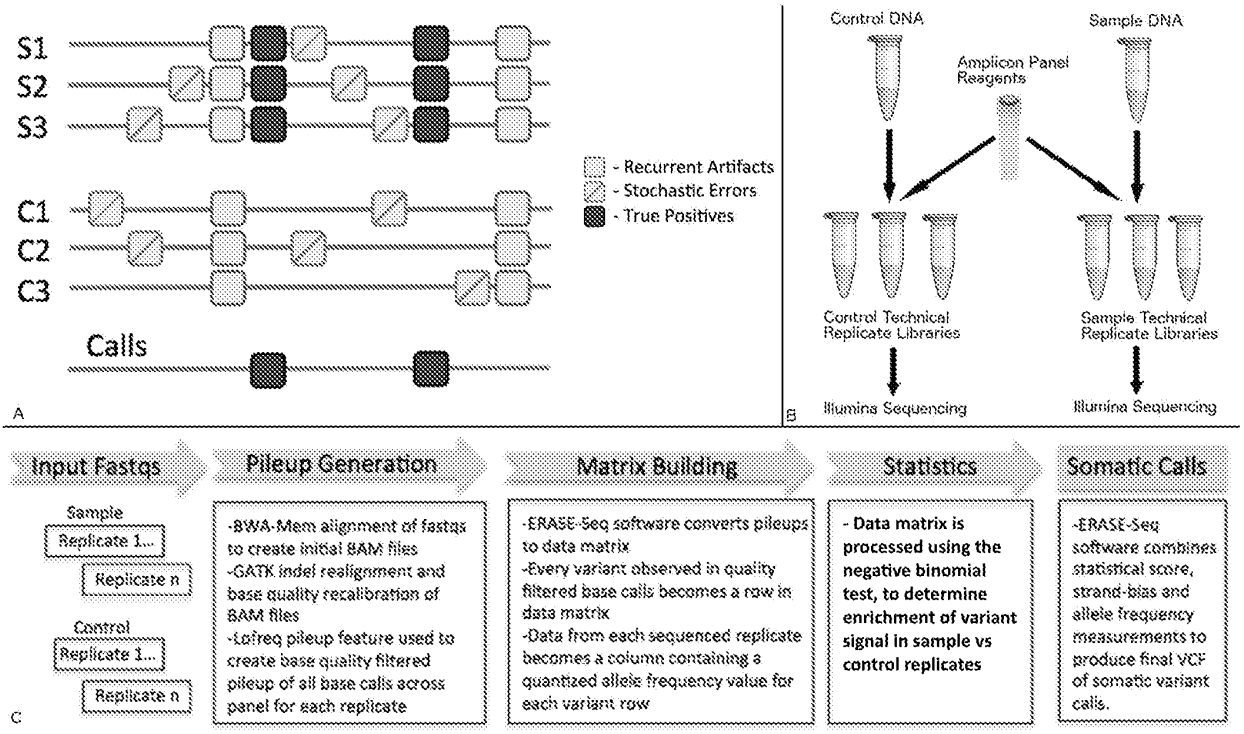


Fig 1. ERASE-Seq concept and method. (A) ERASE-Seq distinguishes true DNA variants from false positives by statistically comparing presence across a series of sample and control technical replicates. False positives arising from recurrent artifacts at error-prone loci (blue squares) are eliminated based on their presence in control replicates. False positives arising from stochastic errors (lined blue squares) are eliminated by inconsistent signal in sample replicates. This allows highly precise detection of true positives (dark blue squares) in final variant calls. (B) The ERASE-Seq molecular workflow is easily applied to amplicon panels by simply preparing and sequencing technical replicates of sample and control DNA in the same fashion they are already being used. Control DNA replicates only need to be generated and sequenced once and can be reused with subsequent samples. (C) The ERASE-Seq bioinformatics workflow begins with BAM file generation and processing of each library replicate. All base calls above a base quality threshold are used to create a pileup for each replicate. ERASE-Seq software converts the replicate pileups to a data matrix representing quantized allele frequencies for each variant in each replicate. The variant data matrix is analyzed using R in order to identify variants that are significantly enriched in sample versus control sequencing runs. These variants are then filtered by strand bias and allele frequency to produce a final set of low frequency somatic variant calls in VCF format.

https://doi.org/10.1371/journal.pone.0195272.g001

show the ability of ERASE-Seq to eliminate these errors. ERASE-Seq represents a significant performance improvement compared to published molecular barcoding strategies sequenced to similar depth and in contrast to molecular barcoding ERASE-Seq can be deployed using existing molecular workflows.

Materials and methods

Analytical sample creation

Spiked samples were created by combining DNA from multiple human cell lines. Whole genomic DNA derived from B-lymphocytes was purchased from Coriell Institute corresponding to samples NA12878 and NA19129. Additionally, whole genomic DNA was extracted from cultured tumor cells lines A549, NCI-H1975, and MDA-MB-231 (ATCC) using the Qiagen DNeasy Blood and Tissue Kit (catalog number 69504) followed by qPCR quantification using the ThermoFisher RNase P assay (catalog number 4316831). NA19129 was used as the background sample into which the other four samples were spiked. Two different spike levels were created based on DNA concentrations. A projected 1% spike sample was created such that

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96% of the DNA mass corresponded to NA19129 and 1% corresponded to each of the four spiked cell lines (S1 Fig). This sample was then diluted 3:1 with NA19129 such that a projected 99% of the DNA corresponded to NA19129 and 0.25% corresponded to each of the four spiked cell lines. A control sample of pure NA19129 was created in order to quantize the error background for ERASE-Seq variant calling. Samples of pure NA12878, MDA-MB-231, NCI-H1975, and A549 were created to determine true variants present in the cell lines.

Library preparation and sequencing

Spiked DNA samples and pure NA19129 DNA were used to create targeted amplicon panel libraries followed by sequencing on Illumina machines. The projected 1% spike sample was used as a template for four replicate multiplex PCR reactions using the 56G oncology panel from Swift Biosciences. Pure NA19129 DNA was also used as a template for four replicate multiplex PCR reactions using the 56G panel. Single replicate libraries were made from pure cell line DNA samples NA12878, MDA-MB-231, NCI-H1975, and A549. Manufacturer's instructions were followed and 10ng of DNA was used as template for each multiplex PCR. These libraries were then sequenced using the MiniSeq 2 x150 high output kit.

The projected 1% spike sample was used as a template for four replicate multiplex PCR reactions using the TST15 oncology panel from Illumina. Pure NA19129 DNA was used as a template for six replicate multiplex PCR reactions using the TST15 panel. Single replicate libraries were made from pure cell line DNA samples NA12878, MDA-MB-231, NCI-H1975, and A549. Manufacturer's instructions were followed and 10ng of DNA was used as template for each multiplex PCR. The TST15 panel consists of 2 tubes so in total 20ng of DNA was used per replicate. Additionally, the projected 0.25% spike was used as a template for four replicate multiplex PCR reactions using the TST15 panel. Manufacturer's instructions were followed with two exceptions. First, the DNA input was doubled from 10 to 20ng per multiplex PCR and the number of cycles was reduced from 16 to 15. Second, the number of cycles in the indexing PCR was reduced from 17 to 14. These libraries were then sequenced using the MiSeq 2 x 300 v3 kit, but reads were stopped at cycle 151 on each end.

A second 0.25% spike sample was created and used as a template for four replicate multiplex PCR reactions using the Spotlight 59 oncology panel from Fluxion Biosciences. Pure NA19129 DNA was also used as a template for eight replicate multiplex PCR reactions using the Spotlight 59 panel. Manufacturer's instructions were followed and 10ng of DNA was used as template for each multiplex PCR. These libraries were then sequenced using the MiSeq 2 x 300 v3 kit, but reads were stopped at cycle 151 on each end.

Raw fastq files for all libraries are available in the NCBI SRA under BioProject Accession Number PRJNA389733.

Expected variant determination

The analytical performance of ERASE-Seq was tested using standards created by spiking DNA from cell lines A549, NCI-H1975, MDA-MB-231, and NA12878 into a background of NA19129. To determine the expected variants across each panel, libraries were made and sequenced using pure cell line DNA as input. This data was analyzed and germline variants were determined for each cell line across each panel. To determine germline variants from sequencing data, quality and strand-bias filtered pileups were searched for variant alleles at >20% allele frequency. This cut-off provided clear resolution between false positives and true germline variants and allowed identification of germline mutations expected at less that 50% allele frequency due to polyploidies found in cancer cell lines. The expected variant set was then defined as loci variant in one or more of the spiked cell lines, but wild-type in the

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NA19129 background. These expected variants were used to assess ERASE-Seq sensitivity and specificity and are listed in S3, S4 and S5 Tables. 1 variant was excluded from analysis using the TST15 panel due to poor coverage (7% of mean).

ERASE-Seq bioinformatics

For the 56G and Spotlight 59 panels primers were trimmed from raw fastq files using Cutadapt[23]. For the TST15 panel primers were trimmed using custom Perl scripts. To test ERASE-Seq performance at lower read depths, down-sampling was performed on fastq files using custom Perl scripts to select a specified number of random reads. We found no performance degradation above a read depth of 5,000x. Trimmed fastq files were aligned to hg19 using BWA-mem[24]. For indel calling, base quality scores were recalibrated and indels realigned using GATK[25]. Lofreq[11] was then used to create pile-ups of all quality-filtered base calls as each position in the panel for all replicates from spike samples and background control samples. Custom Perl scripts were then used to parse pile-up data into a matrix containing read counts for each sample and control replicate at each possible variant. The data matrix was then processed in R using the DESeq[26] negative binomial test to quantify the significance of enrichment between variant count observations in sample and control replicates.

ERASE-Seq exploits the DESeq statistical framework in order to model the probability that a variant exists in sample versus control data. This framework has been demonstrated to provide high resolution in gene expression and copy number studies by accurately integrating replicates from both treated and untreated groups and modeling shot noise present in NGS count data using the negative binomial distribution[26]. A major innovation of ERASE-Seq is to convert replicate variant call data into a matrix than can use this statistical framework, allowing variant calls to be made based on a statistically robust integration of both intrasample technical variation and intersample background artifacts.

ERASE-Seq uses this model to assign a p-value to each possible sample variant call as using the DESeq method previously described[26] For each possible variant if the p-value is above a cutoff/threshold α, then the null hypothesis is true and there is no mutation present in the sample as compared to control runs. Conversely, if the null hypothesis is false, p-value $< \alpha$, then a mutation call is made for the variant in question. Whereas previously DESeq uses a p-value threshold to determine the presence of a significant copy difference between sample and control runs, here we use a similarly derived p-value threshold to determine the presence of a mutated copy in the starting DNA sample with respect to controls runs.

Subsequently the Benjamini and Hochberg procedure[27] is applied to generate a multiple hypothesis-adjusted p-value to correct for false positives that arise from performing the tens to hundreds of thousands of variant tests across the complete amplicon panel space. The final multiple hypothesis-adjusted p-value adjP_i forms the basis for mutation identification. Custom Perl scripts then integrate adjP_i with strand-bias and allele frequency measurements to produce a final set of variant calls. Allele frequencies for each variant are calculated by taking the mean of allele frequencies observed across all replicates. The strand bias measure used was (AF strand 1/AF strand 2), called Strand Bias Factor. When this factor deviates from 1 in either direction that is indicative of strand bias. In our experiments variants exhibiting a Strand Bias Factor of magnitude > 5 were excluded. Precise adjP_i and allele frequency thresholds used depend on panel and desired limit of detection.

Sensitivity and specificity calculations

Sensitivity, specificity, and false positive rate are used to compare the performance of ERASE-Seq with several published low frequency DNA variant detection approaches. These metrics

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Table 1. ERASE-Seq performance comparisons.

Detection Method	Method Basis	Allele Frequency Range	Sensitivity	False Positives per 10k Variant Tests
ERASE-Seq Spotlight 59	Replicates and background modeling	0.1–0.5%	94%	0.1
ERASE-Seq TST15	Replicates and background modeling	0.07–1.3%	94%	0.7
Integrated Digital Error Suppression [17]	Duplex molecular barcoding and background modeling	0.05–1.6%	97%	<36
Digital Sequencing [12]	Molecular barcoding and background modeling	0.1–1%	66%	not reported
Amplicon Molecular Barcoding [16]	Molecular barcoding only	1–2%	85%	0.2
Molecular Inversion Probes [15]	Molecular barcoding only	0.75–1.5%	72%	0.1

https://doi.org/10.1371/journal.pone.0195272.t001

are ideal because, unlike other measures like Positive Predictive Value, they are not dependent on the number of true positives in the test sample and thus objectively quantify the performance of the method itself. Sensitivity, or true positive rate, is defined as (True Positives)/(True Positives + False Negatives) and expresses the ratio of known variants in the analytical sample that were called by the method. Specificity is defined as (True Negatives)/(True Negatives + False Positives), and expresses the ratio of alleles known not to be present in the sample that are called as such. In some of the data we chose to instead show false positive rate, which is 1- Specificity. This can also be defined as (False Positives)/(False Positives + True Negatives) and represents the average likelihood that a variant will be called as present in the sample when it is in fact absent. This is alternatively expressed as false positive calls per 10,000 variant tests (False Positive Rate *10,000). These calculations were performed based on our own data and compared to similarly computed metrics from published data to compare the techniques listed in Table 1 and S1 Table.

ERASE-Seq is compared to molecular barcoding methods that have been implemented over large target regions and have published performance metrics using analytical standards. Methods that use barcoding alone begin to lose resolution below 1% allele frequency. Barcoding methods that incorporate background modeling can provide sensitivity in the ultralow range but do not demonstrate high specificity. Technical replication allows ERASE-Seq to provide high sensitivity and specificity in the ultralow allele frequency range.

ERASE-Seq, Lofreq2, and some of the molecular barcoding papers analyzed in this study report numbers of false positives across the entire panel. In these cases, the total number of variant tests used for our false positive rate calculations is equal to (Total Number of Base Pairs in Panel)*(3 SNV Tests + 1 Indel Test). This is an approximation because the number of indel tests performed varies by data set. False positive rate metrics are ideal because they characterize the overall number of false positives that will be called per set number of variants considered. If not normalized in this way, the absolute number of false positives will always depend on both the overall panel size and the total number of possible variants considered (variant tests). For example, in the analytical data reported for the iDES [12] method a set of only 279 preselected negative control variants is tested (as opposed to all variants across the entire panel) to determine their specificity, so in our comparison we use 279 as the number of variant tests to determine their false positive rate. Data used to calculate false positive rates and sensitivity for the techniques listed in Table 1 and S1 Table can be found in the following sources: Peng et al: Table 1, Hiatt et al: Fig 4, Newman et al (2016): Fig 4a, Lanman et al: Results; Analytic specificity and sensitivity.

Data for Lofreq2 was obtained by using the same bam files used for ERASE-Seq analysis. These files were recalibrated for base quality and the indels were realigned according to GATK best practices[25]. SNVs and indels were called using default lofreq2 parameters with a

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homopolymer limit of three. Output vcfs were subsequently tested for sensitivity and false positive rate.

Results

Sequencing error composition

Replicate experiments against the ERASE-Seq background model allow for an accurate categorization of false positive variant calls observed in sequencing data as either recurrent artifacts or stochastic errors. Fig 2C and 2D shows all false positive calls present in sequencing reads filtered by base quality, read depth, and strand bias for 56G and TST15 experiments. Recurrent artifacts are those false positive calls present in the single replicate data but eliminated by ERASE-Seq due to their presence in the background model. Stochastic errors are those false positives called in single replicate ERASE-Seq, but eliminated with increasing replicate number. False positives are binned by allele frequency and divided into recurrent artifacts and stochastic errors (Fig 2C and 2D). The data show that most false positives are recurrent artifacts at all allele frequencies, with 71% of 56G false positives and 95% of TST15 false positives belonging to this category.

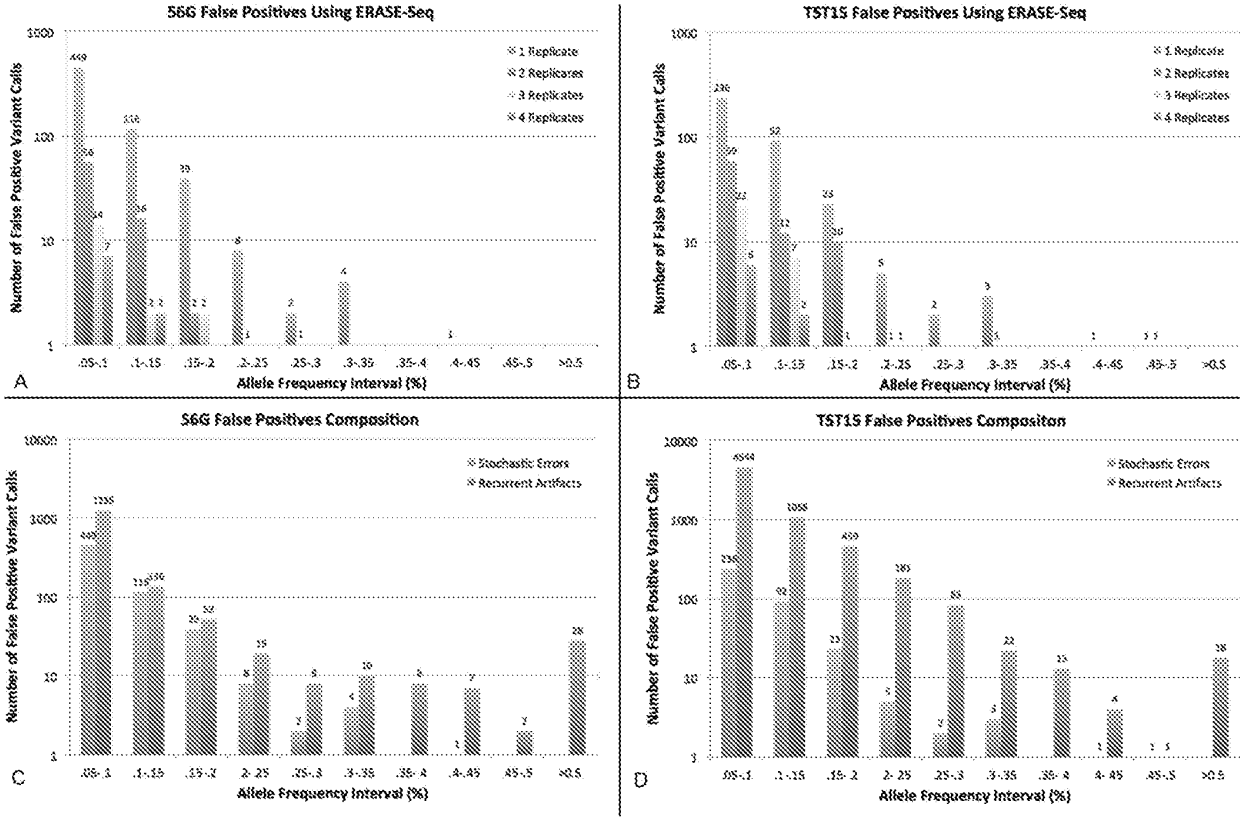


Fig 2. False positive composition. (A,B) The number of false positive calls in 0.05% allele frequency intervals is shown for ERASE-Seq using 1, 2, 3, and 4 replicates for the amplicon panels 56G and TST15. (C,D) The number of false positives using standard intra-sample variant calling metrics (base-quality, strand-bias and read-depth filters) are shown in 0.05% allele frequency intervals for 56G and TST15. They are further divided into recurrent artifacts and stochastic errors. Stochastic errors are those called in single replicate ERASE-Seq and recurrent artifacts are those eliminated in single replicate ERASE-Seq based on the background model.

https://doi.org/10.1371/journal.pone.0195272.g002

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Both types of errors decline in abundance as allele frequency increases although recurrent artifacts are more persistent than stochastic errors. Stochastic errors largely disappear above 0.3% allele frequency and are completely absent above 0.5% in both data sets whereas numerous recurrent artifacts remain in the data above 0.5% allele frequency. Notably, most recurrent artifacts above 0.5% are indels, whereas the majority below 0.5% are SNVs. The general trends observed are the same for both panels but each has its own error profile. TST15 has a 3-4x higher number or recurrent artifacts, but only about ½ of the stochastic errors in AF ranges below 0.2%. This suggests some panel specific optimization of the error suppression protocols is possible. In our experiments detailed below these unique error profiles did not translate into significant differences in ERASE-Seq performance. Spotlight 59 error profiles were not analyzed however the panel chemistry and content is highly overlapping with 56G such that similar results are expected.

ERASE-Seq eliminates ultralow frequency errors via technical replication

To achieve high specificity at AF $< 0.2\%$, ERASE-Seq uses the statistical power of technical replication. Fig 2A and 2B shows the number of false positives in analytical samples using ERASE-Seq and leveraging 1, 2, 3, and 4 sample technical replicates for both 56G (A) and TST15 (B). False positive counts by bin are shown between 0.05% and 0.5% AF. Robust elimination of false positives at $> 0.5\%$ allele frequency occurs due to the background-aware caller, for even a single sample replicate. Below 0.5% the number of false positives increases with decreasing allele frequency, up to hundreds of false positive calls for single replicate samples in the 0.05–0.1% interval. Increasing replicate number maintains high specificity at ultralow allele frequencies. Adding a second sample replicate eliminates 88% of the errors observed with a single replicate for the 56G panel and 77% for the TST15 panel. We have settled on a maximum of 4 replicates, because at 10ng input per replicate this requires a total of 40ng, a quantity obtainable from cfDNA samples isolations. Higher replicate numbers are not feasible, but great results can still be obtained from 2 replicate experiments where input material is limited. Using four replicates allows precise calling down to the lowest allele frequencies. On the TST15 panel only 2 false positives out of 120469 possible variants are called above 0.1% (specificity = 99.9983%) and 8 false positives are called above 0.05% (specificity = 99.9933%). Similarly, on the 56G panel only 2 false positives out of 94888 variant tests are called above 0.1% (specificity = 99.9979%) and 9 false positives are called above 0.05% (specificity = 99.9905%).

ERASE-Seq performance

ERASE-Seq eliminated the clear majority of sequencing errors and enabled accurate variant calling to sub 0.1% allele frequency. Fig 3 shows allele frequency measurements and ERASE-Seq p-values plotted for observed variants in three different low frequency spike-in analytical experiments. In the top panel observed variants are shown by allele frequency from a single replicate and true positives are buried among noise from sequencing errors, making accurate calling impossible. The bottom panel shows the same variants' ERASE-Seq p-value enabling differentiation between true and false positives. The p-value measurement can differentiate all true variants tested from false positives with 100% sensitivity and specificity in the two low frequency experiments (Fig 3A, 3B, 3D and 3E). Ultralow frequency variants shown in the third experiment are still detected with high sensitivity and specificity down to 0.07% allele frequency (Fig 3C and 3F).

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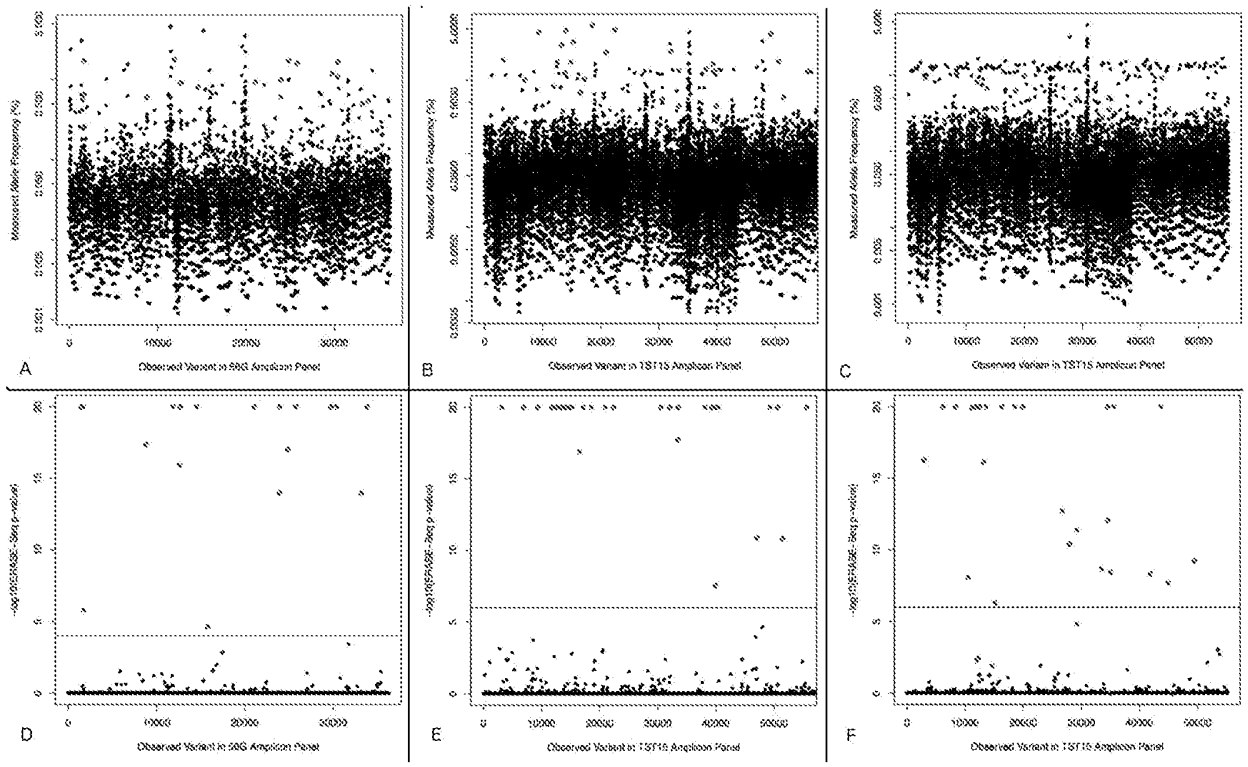


Fig 3. Error reduction using ERASE-Seq. Low frequency variants observed in three analytical DNA spikes mixtures are shown both by allele frequency in the top panel and by ERASE-Seq multiple hypothesis adjusted p-value in the bottom panel. True positives are shown in red and noise is shown in black. (A,D) A spiked DNA mixture is analyzed using the Swift Biosciences 56G amplicon panel. The 19 snvs and one indel ranging from 0.27–1.78% expected allele frequency are detected with perfect sensitivity and specificity using ERASE-Seq. (B,E) A spiked DNA mixture is analyzed using the Illuimina TruSight 15 amplicon panel. The 30 snvs and one indel ranging from 0.35–5.6% expected allele frequency are detected with perfect sensitivity and specificity using ERASE-Seq. (C,F) A more challenging spiked DNA mixture is analyzed using the Illuimina TruSight 15 amplicon panel. The 30 snvs and one indel range from 0.07–1.3% expected allele frequency. All variants above 0.3% allele frequency are detected with perfect sensitivity and specificity and robust detection of ultra-low frequency alleles is achieved with a small number of false positives.

https://doi.org/10.1371/journal.pone.0195272.g003

Performance comparisons

ERASE-Seq provides superior variant calling performance compared to previously described low frequency detection methods at similar read depth and DNA inputs (Table 1, S1 Table). Table 1 compares the performance of ERASE-Seq to several other studies that use analytical spike samples to establish performance metrics. In these experiments, DNA with known variants was spiked into wild-type DNA at low relative frequency and libraries made from the spiked sample were sequenced. ERASE-Seq strongly outperforms algorithms based on base quality scores such as Lofreq2[11]. ERASE-Seq is also compared to published strategies using molecular barcoding[15, 16]. Peng et al. and other published methods lose sensitivity and specificity below 1% allele frequency whereas ERASE-Seq maintains perfect sensitivity and specificity to 0.3% allele frequency using both the TST15 and 56G panel. An ultralow frequency analytical spike containing multiple variants below 0.1% allele frequency was also analyzed using the ERASE-Seq. This sample shows 94% total sensitivity and identifies 3/3 variants below 0.1% allele frequency while maintaining a false positive rate of less than 0.007% of variant tests using the TST15 panel. Additionally, an ultralow spike containing variants between 0.1% and 0.5% analyzed using ERASE-Seq with the Spotlight 59 panel demonstrated 94%

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sensitivity and a false positive rate of less than 0.001%. This demonstrates a large improvement over Guardant digital sequencing which only achieves 66% sensitivity for variants between 0.1–1% allele frequency and does not publish a false positive rate[12]. The method with comparable sensitivity to ERASE-Seq in the ultralow allele frequency range is iDES[17]. However, iDES specificity measurements are taken from an extremely limited search space, testing only 279 preselected negative control variants throughout the panel thus only demonstrating a false positive rate as low as 0.358%. In contrast, ERASE-Seq remains highly specific across the 120,469 variant tests comprising the entire TST15 panel, demonstrating a false positive rate of 0.0064% when calling variants to 0.05% allele frequency. Similarly, ERASE-Seq remains highly specific across the 100,904 variant tests comprising the entire Spotlight 59 panel, demonstrating a false positive rate of 0.00105% when calling variants to 0.1% allele frequency.

Single replicate ERASE-Seq performance

Applying ERASE-Seq to single replicate data provides a background aware variant caller that can be used for typical data generated by targeted sequencing. This analysis demonstrates both the advantages provided by the ERASE-Seq background model and the limitations of variant calling without technical replicates. The same sequencing data used for ERASE-Seq was also analyzed with Lofreq2 (Fig 4). Sensitivity and specificity were measured using the two algorithms across four TST15 samples and four 56G samples, with the same fastq data inputs. The background model-driven ERASE-Seq variant calling algorithm outperforms the base quality score-driven Lofreq2 algorithm. In Fig 4 sensitivity is shown for variants expected between 0.3 and 1% allele frequency, of which each panel contains ten. False positive rates are also shown. ERASE-Seq provides improved sensitivity and reduced false positive rate in all samples analyzed. When compared to Lofreq2, single replicate ERASE-Seq provides an increase in average sensitivity from 71% to 93% and an average 6-fold decrease in the false positive rate (from about 2 down to about 0.3 FPs per 10k variants).

Particularly dramatic gains are achieved for indel calls that are relatively abundant above 0.3% and are recurrent in nature. (Fig 2A and 2B). However, in the space below 0.3% significant stochastic errors exist that cannot be eliminated without replication.

Practical considerations for ERASE-Seq implementation

Single replicate ERASE-Seq can be applied to existing data sets by creating a background error model to provide excellent performance down to AFs of 0.5%. Its sensitivity is above 90% and specificity is very high, with a false positive rate below 0.3FP/10k variants.

The ideal implementation of ERASE-Seq uses multiple technical replicates sequenced to a read depth of at least 5,000x. The overall depth per sample (at least 20,000x) is not a major challenge, as these depths are commonly achieved in targeted deep sequencing (S1 Table). When using off the shelf reagents, two practical challenges are the additional material costs per targeted amplification reaction and the higher DNA input requirements. These challenges can be addressed by purpose-built kits like Spotlight 59 that provide all the targeted amplification and library preparation reagents necessary for 4 replicates and draw on optimized amplicon chemistries that minimize DNA input and overall reaction tube number. Regardless of the replicate number and amplicon panel chosen, the background needs to only be produced once and can be reused for each sample set.

Discussion

ERASE-Seq brings together the principles of deconvolution and technical replication to achieve improved performance in ultralow frequency DNA variant calling. The deconvolution

EX1002.800

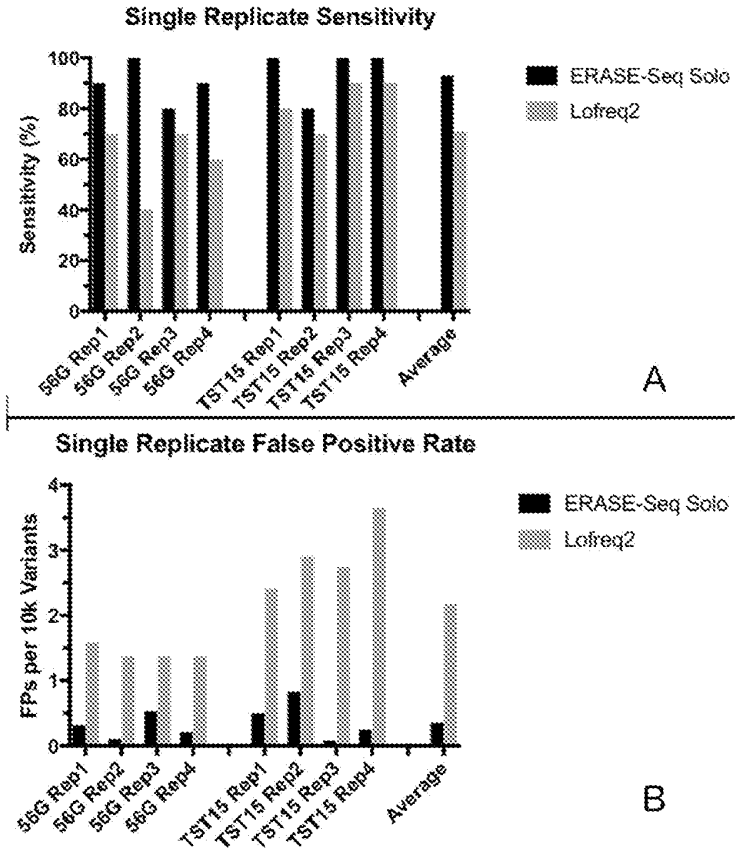


Fig 4. Single replicate ERASE-Seq performance. The ERASE-Seq algorithm may also be used with single replicates to eliminate false positives resulting from recurrent artifacts. This fig demonstrates ERASE Seq's large gains in resolution below 1% allele frequency as compared to Lofreq2, a high-performing standard low frequency calling algorithm that does not model background errors and therefore does not eliminate recurrent artifacts. Sensitivity in the 0.3–1% allele frequency range is shown along with false positive rate for four analytical samples using the TST15 amplicon panel and four analytical samples using the 56G amplicon panel. ERASE-Seq provides an average increase in sensitivity from 71% to 93% and a greater than six-fold reduction in false positive rate as compared to Lofreq2.

https://doi.org/10.1371/journal.pone.0195272.g004

component consists of performing repeated library preparation and sequencing of negative control DNA. Each variant in the panel is then quantified across the negative control replicates to establish a variant-specific background noise model. Actual sample measurements are then tested for statistically significant enrichment against the background model to distinguish true positive variants from noise. Previous studies have employed similar deconvolution strategies to increase precision[4, 28] but this is the first background-aware caller to incorporate sample replicates into a robust statistical variant calling model.

ERASE-Seq's unique approach is to incorporate multiple sample measurements into the statistical test between variant counts in background and sample replicate data. It has been shown that the high degree of noise in NGS data can be mitigated through technical replication and this has been widely adopted in gene-expression studies[29, 30] and suggested for variant calling[9]. ERASE-Seq provides a quantitative statistical model for using technical replicates that is applicable to ultralow frequency somatic variant discovery and provides significant increases in accuracy.

EX1002.801

Our data demonstrate the practical power of both the background modeling and replication components of ERASE-Seq. A performance comparison between ERASE-Seq (single replicate) and Lofreq2 applied to the same data set measures the gains of the background-aware caller alone, without use of replicates (Fig 4). Base quality score-driven caller algorithms such as Lofreq2 begin to show false positives below 1% allele frequency[11]. However, the sub 1% variant space is of high interest in clinical liquid biopsy applications[8, 12, 17], so we compare variant calls in the 0.3–1% allele frequency range for single replicate ERASE-Seq. We show improvements in specificity in all eight samples tested while reducing false positive rates by an average of greater than six-fold (Fig 4). These gains are due to the elimination of recurrent artifacts only. Notably, the Lofreq2 caller is equipped with a homopolymer filter that was used in this analysis and was unable to match the power of ERASE-Seq's empirical background error model for false positive elimination. The variant calling performance metrics are corroborated by our false positive component analysis (Fig 2C and 2D) that demonstrates the clear majority of sequencing errors above 0.3% allele frequency are recurrent artifacts. This also demonstrates the ability of ERASE-Seq to provide large gains to molecular pathology laboratories without altering bench protocols. Laboratories may create and sequence negative control libraries using their current panel reagents. Subsequently sample library preparation and sequencing continue unchanged and resolution can be gained by simply employing the ERASE-Seq variant caller.

Technical replication enables the highest precision in ERASE-Seq experiments. Our false positive component analysis (Fig 2C and 2D) demonstrates stochastic errors begin to accumulate in abundance at ultralow frequencies when using single replicate data. This is because polymerase errors[13, 31] populate the sequencing libraries at ultralow frequencies across the large sequence spaces assayed. These stochastic errors are recalcitrant to elimination because they are present in the sequencing library[9]. Both molecular barcoding and ERASE-Seq technical replication attempt to address this challenge. Replication allows for accurate discernment of the alleles in the biosample whereas base quality-driven algorithms only allow for accurate discernment of alleles present in the sequencing library. The power of technical replication is what gives ERASE-Seq unparalleled specificity in the sub 0.1% allele frequency range, while maintaining high sensitivity. In fact, the sensitivity of the ERASE-Seq method is not limited by inherent background noise, but rather DNA input copy number. For example, 10ng of human DNA input should contain approximately 3333 copies of each allele, meaning that a 0.1% variant will have on average 3 copies. Below this allele frequency variant incorporation in all four replicates becomes inconsistent, following a Poisson distribution. We show that sensitivity in this range can be retained by increasing DNA input to 20ng per replicate by successfully detecting 3/3 sub 0.1% variants on the TST15 panel (S1 Table).

The wide applicability of ERASE-Seq is demonstrated by successful use of this method with three different oncology amplicon panels and two different DNA sample types. The performance gains observed are consistent across the panels tested showing the robustness of ERASE-Seq. Over 90% sensitivity down to AFs below 0.1% was obtained while maintaining extremely low false positive rates. Perfect sensitivity and specificity is demonstrated down to 0.3% allele frequency. The false positive profiles are highly consistent as is the performance of single replicates. In addition, experiments show high correlation between observed and expected allele frequencies (Fig 5, S2 Table) and training parameters remain robust over repeated analytical tests.

A key implementation question is the applicability of error backgrounds obtained using gDNA standards across other sample types. We have addressed this by analyzing an external data set using the Horizon cfDNA standard consisting of fragmented DNA that aims to replicate cfDNA patient samples, and TST15. The data (Fig 6) demonstrates a slight advantage

EX1002.802

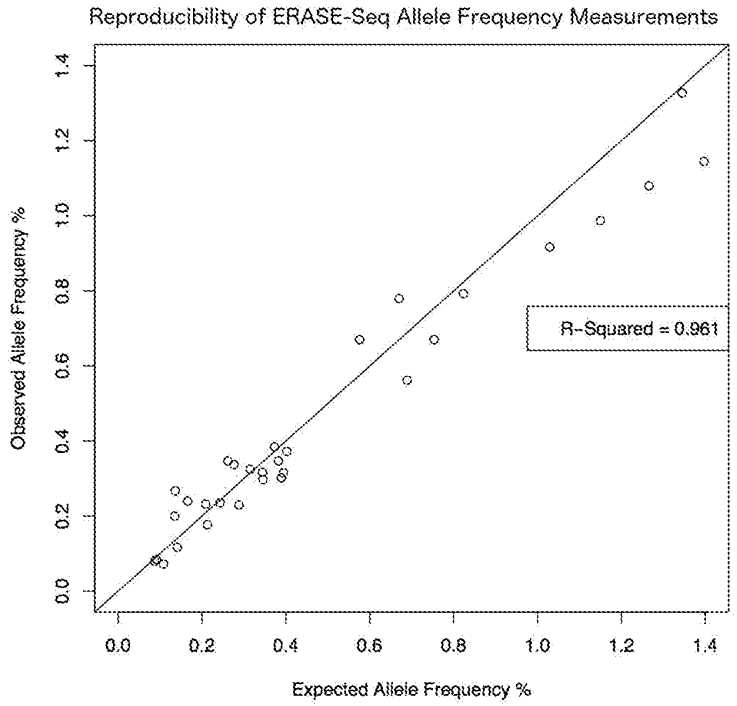


Fig 5. Observed vs expected allele frequencies. ERASE-Seq demonstrates high reproducibility (R-squared = 0.961) in allele frequency determination between experiments, even in the ultralow allele frequency range. This graph compares measured allele frequencies between the 1% TST15 spike and the 0.25% TST15 spike. The 0.25% spike is a simple 4X dilution of the 1% spike into the same NA19129 DNA background so variant allele frequencies in the 0.25% spike are expected to be ¼ their value in the 1% spike. The y-axis plots observed variant allele frequencies in the 0.25% spike and the x-axis plots their expected values.

https://doi.org/10.1371/journal.pone.0195272.g005

when also using fragmented cfDNA, but the gains are similar in order of magnitude when using our standard gDNA derived background. False positive rates are summarized in S2 Fig and S6 Table. This data set is important in demonstrating the ERASE-Seq approach to be highly efficacious even when different sample types (gDNA vs fragmented DNA) are used to generate the position-specific background model. It demonstrates that the vast majority of errors are introduced during targeted amplification, library preparation and sequencing. The nature of the starting sample only drives a small minority of errors, accounting for less than 5% of false positive calls in our analytical experiment (Fig 6).

ERASE-Seq strongly outperforms molecular barcoding strategies at similar read depths and inputs (Table 1). iDES, a recently developed method that combines a background-aware caller with molecular barcoding, demonstrated great sensitivity in the ultralow frequency range but without the high panel-wide specificity of ERASE-Seq. The iDES approach used a very limited search space (279 variants) to limit false positive calls. This approach to reducing FP rates can be very useful clinically if only a subset of less than a few hundred variants are relevant to disease assessment, but suffers from clear limitations when analyzing wider mutation spectra. Another commonly employed cfDNA assay, the Guardant 360 test also employs a combination of molecular barcoding and background modeling to increase performance[12]. Previously published analytical data for Guardant 360 demonstrate a sensitivity of 66% for variants present between 0.1–1%[12] as compared to over 90% for both iDES and ERASE-Seq[12, 31].

EX1002.803

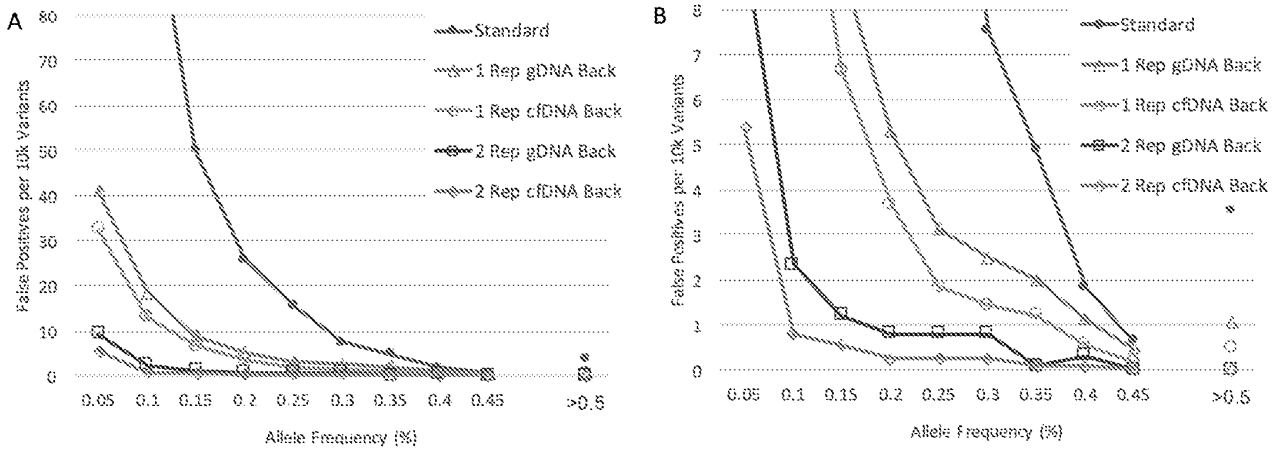


Fig 6. Robustness of the ERASE-Seq approach across different sample types. We analyzed a previously produced data set looking at a Horizon cfDNA standard spike (fragmented DNA) using both an unrelated gDNA background standard and a more similar Horizon cfDNA standard. The false positive rate per 10,000 variant tests is plotted for all conditions. ERASE-Seq results from applying a background model using either background (empty triangle, circle) show a high reduction in the false positive rate for both as compared to a standard caller (filled round). Of the two, using a similar Horizon cfDNA background (empty circles) provides slightly better error correction, while both perform very well above 0.5% allele frequency. The same relationship holds when using two replicates for the Horizon cfDNA sample (square, rhombus), with very low false positive rates above 0.2%. Together, the data demonstrate consistent performance of the background model across sample types. A summary of the false positive rate dependence on the replicate number and control background data used is shown in S6 Table.

https://doi.org/10.1371/journal.pone.0195272.g006

The greatest loss in sensitivity is at the 0.1% level, where only 33% of variants are detected. Analytical specificity of the test is not reported below AF values of 2.5%, nor are the bioinformatics details describing how calls are made[12]. It is likely that search space reduction is also necessary to decrease the false positive rate.

Many recent publications[6–8, 12, 17] report that somatic variants in cancer patient cfDNA are commonly found in the 0.05 to 0.5% range, so increases in sensitivity and specificity will significantly impact the effectiveness of these assays. Even in the advanced patient populations (predominantly stage IV patients), about a third of samples tested do not detect any somatic variants when using the Guardant 360 test[12]. Several recent publications looking and this and other tests challenges in demonstrating high concordance to solid tumors and agreement between different tests[18–20, 32]. This is likely due to a combination of low cfDNA content in these samples and assay sensitivity limitations, so improved sensitivity/specificity will directly result in liquid biopsies being applicable to a larger percentage of patients, earlier in the disease process, with fewer false positives.

Search space reduction can also be combined with ERASE-Seq with the advantage of inherently lower false positive numbers, but we chose not to take this approach. Because the list of clinically relevant mutations is evolving quickly we believe that a whole panel caller is superior. Additionally, there are situations where the high specificity of ERASE-Seq is paramount. Tumor suppressor genes including TP53[33], RB1[34], and Notch1[35] have been shown to have wide loss of function mutation spectra distributed over multiple domains and to include missense, nonsense, and frameshift subtypes. Coupled with the observation that many cancer types can arise from loss of function in one of several tumor suppressor genes[36, 37], the ability to query larger sequence spaces is necessary to avoid false negative clinical results. The analytical work here presents a versatile platform for high-resolution detection of low frequency DNA variants that is particularly suited to the expected allele frequencies found in liquid

EX1002.804

biopsy samples. Furthermore, ERASE-Seq is the first ultralow allele frequency DNA variant detection method that does not require the adoption of new molecular reagents, making it easily implementable for research and clinical laboratories.

Conclusions

Liquid biopsy testing methods are being developed at an rapid pace due to the promise of the technology to better represent the diversity of solid tumors and their ability to follow disease progression longitudinally and noninvasively. While a number of recently developed targeted panels and clinical tests have shown great results in selected advanced patient populations, recent literature also highlights significant limitations. Limited sensitivity and specificity have led to measurements of low concordance for CLIA approved cfDNA tests [20] and low sensitivity to solid tumor mutations [32]. Further performance improvements are needed to address a patient populations where at a majority of variants are in the 0.1–1% allele frequency range. Here we present analytical validation data for ERASE-Seq, a new method that, instead of using molecular barcodes, uses technical replicate analysis and a sequencing noise background model to make calls. Using analytical standards on par with previously described methods and clinically realistic DNA input quantities, we detect SNVs and indels between 0.05% and 1% allele frequency with greater than 90% sensitivity and a false positive rate below 0.1 calls per 10,000 possible variants. This provides a 10 to 100-fold reduction in false positive rates compared to published molecular barcoding methods. Future work in patient samples will evaluate clinical improvement by looking at concordance to tumor biopsies and orthogonal high sensitivity PCR-based methods in cfDNA.

Supporting information

S1 Fig. Schematic of analytical sample creation. DNA from four different cell lines was spiked into a normal background (NA19129) at a target of 1% per cell line. Variant allele frequencies present in the analytical mixture spanned a wide range (0.25–5.6%) due to the fact than many variants were shared across some or all cell lines, some variants had non-standard ploidies due to copy number variation, and different quantitation methods led to slightly different input quantities of each cell line relative to the background. This created an analytical sample with many low frequency variants that could be used to test multiple panels. The analytical sample pictured could be further diluted with NA19129 DNA to create samples containing ultralow allele frequency variants.
(TIFF)

S2 Fig. Differences in noise between Horizon cfDNA and gDNA samples. The analysis of Horizon cfDNA standards and gDNA allows to compare the noise false positive rate (FP rate) dependence on sample type and DNA fragmentation. Both standard analysis (filled symbols) and ERASE-Seq one replicate only data show a higher noise level for cfDNA as compared to gDNA derived data.
(TIFF)

S1 Table. Detailed ERASE-Seq performance comparisons. ERASE-Seq is compared to other low frequency variant detection methods. Performance in terms of sensitivity and specificity is reported in conjunction with sequence depth and DNA input quantity. At comparable sequencing depths and DNA inputs ERASE-Seq outperforms barcoding methods in terms of both sensitivity and specificity. Additionally, ERASE-Seq utilizes moderate increases in sequencing depth to strongly outperform other methods in the ultralow allele frequency

EX1002.805

range. The * indicates a two-tube multiplexed PCR amplicon panel which is why DNA inputs are higher.
(DOCX)

S2 Table. TST15 variant allele frequencies. This table shows the spiked variants along with their allele frequencies across the TST15 panel for the 1% and 0.25% spikes along with the expected allele frequencies of the 0.25% spike. The 0.25% spike is a simple 4X dilution of the 1% spike into the same NA19129 DNA background so variant allele frequencies in the 0.25% spike are expected to be ¼ their value in the 1% spike.
(DOCX)

S3 Table. Expected variants in the TST15 panel region. Expected variants in the spiked analytical mixtures are shown along with their allele frequency in each pure spiked cell line. In total 31 variants, 30 SNVs and one insertion, compose the test set. All variants were homozygous for the hg19 reference allele in the pure NA19129 background.
(DOCX)

S4 Table. Expected variants in the 56G panel region. Expected variants in the spiked analytical mixtures are shown along with their allele frequency in each pure spiked cell line. In total 20 variants, 19 SNVs and one deletion, compose the test set. All variants were homozygous for the hg19 reference allele in the pure NA19129 background.
(DOCX)

S5 Table. Expected variants in the Spotlight 59 panel region. Expected variants in the spiked analytical mixtures are shown along with their allele frequency in each pure spiked cell line. In total 18 variants, 17 SNVs and one deletion, compose the test set. All variants were homozygous for the hg19 reference allele in the pure NA19129 background.
(DOCX)

S6 Table. ERASE-Seq efficacy depending on sample type, replicate number and background type. False positives per 10,000 variants tested is shown for ERASE-Seq implementations using a variety of analytical sample types (fragmented cfDNA standard vs gDNA) and background models used. Replicates provide the lowest false positive rates, and function very well independent of sample type.
(DOCX)

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Formal analysis: Nick Kamps-Hughes.

Project administration: Cristian Ionescu-Zanetti.

Resources: Laurie Kurihara, Timothy T. Harkins, Prithwish Pal, Claire Ray.

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Writing – review & editing: Andrew McUsic, Laurie Kurihara, Timothy T. Harkins, Prithwish Pal, Claire Ray, Cristian Ionescu-Zanetti.

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Lennon et al. Genome Medicine (2016) 8:112
DOI 10.1186/s13073-016-0370-4

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Technological considerations for genome-guided diagnosis and management of cancer

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Abstract

Technological, methodological, and analytical advances continue to improve the resolution of our view into the cancer genome, even as we discover ways to carry out analyses at greater distances from the primary tumor sites. These advances are finally making the integration of cancer genomic profiling into clinical practice feasible. Formalin fixation and paraffin embedding, which has long been the default pathological biopsy medium, is now being supplemented with liquid biopsy as a means to profile the cancer genomes of patients. At each stage of the genomic data generation process—sample collection, preservation, storage, extraction, library construction, sequencing, and variant calling—there are variables that impact the sensitivity and specificity of the analytical result and the clinical utility of the test. These variables include sample degradation, low yields of nucleic acid, and low variant allele fractions (proportions of assayed molecules carrying variant allele(s)). We review here the most common pre-analytical and analytical factors relating to routine cancer patient genome profiling, some solutions to common challenges, and the major sample preparation and sequencing technology choices available today.

Background

Technologies that profile the cancer genome are powerful tools to elucidate molecular mechanisms that contribute to the pathogenesis, progression, regression, and resistance of neoplastic disease [1]. Over the past 5 years, our understanding of these mechanisms has improved, in part due to projects such as The Cancer Genome Atlas (TCGA) [2]. Accordingly, applications for tumor molecular profiling have become increasingly translational. Genomic testing of patient tumors is now used in diagnostics [3], precision therapy selection [4], disease progression monitoring (mostly in a clinical research setting) [5], and clinical trial enrolment [6]. However, mapping the cancer genome is not a simple task. Each individual's cancer genome contains a multitude of alterations and alteration types (for example, single base changes, structural variation, epigenetic changes) that require specific wet lab and analytical approaches for optimal performance of genomic profiling.

Profiling the cancer genome of a patient sample is complex and fraught with opportunities for technical artifacts, reduced sensitivity, false-positive findings, and outright test failure. Annotation, interpretation, and reporting of clinically relevant variants encompass the process by which genomic data are translated into the practice of medicine. At each of the steps to produce genomic data—sample collection, nucleic acid extraction, library preparation, sequencing, and variant calling—one must consider how technical and methodological decisions might impact the sensitivity and specificity of the data that will be delivered to a clinician for the provision of patient care. We present here a review of the major technical considerations, test selection considerations, sequencing technologies, and analytical variables that impact cancer genomics.

Pre-analytical considerations

Sample collection, preservation, and manipulation are important pre-analytical factors to consider prior to genomic data generation (Fig. 1). Traditional methods for tumor biopsy include fine- or core-needle aspiration or surgical resection. Formalin fixation and paraffin embedding (FFPE) is most often used for sample preservation though fresh frozen tissue or disaggregated cells are sometimes used for specific downstream applications. Recently, liquid biopsy has emerged as a potentially powerful and minimally invasive alternative for routine

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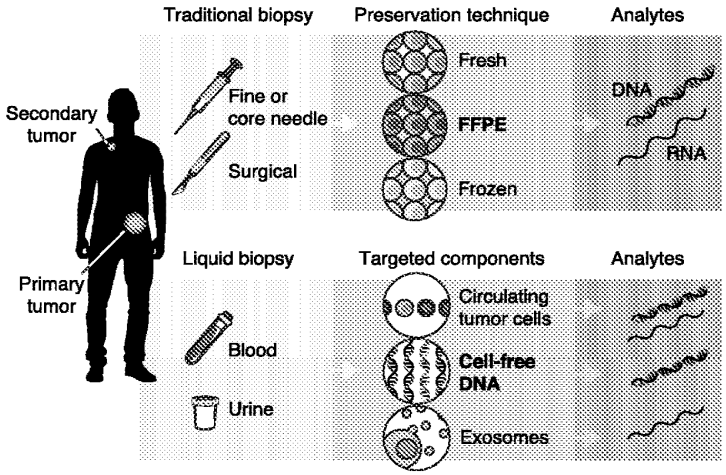


Fig. 1 Overview of the most commonly used biopsy techniques, preservation methods, and genomic analytes. Traditional biopsy methods include fine- or core-needle biopsy or surgical resection. These biopsies typically only access the primary tumor site. From traditional tissue biopsy the most common pathological preservation path is through formalin fixation and paraffin embedding (FFPE), though fresh frozen tissue or disaggregated cells are sometimes also available. From each of these material types, both DNA and RNA can be extracted. Liquid biopsy usually involves blood draw, though some groups are now testing urine and other body fluids. Liquid biopsy can have representative somatic lesions from more than one tumor site. Circulating tumor cells (CTCs), cell-free DNA (cfDNA), and exosomes or extracellular vesicles (EVs) are the most common components of liquid biopsy that are isolated for somatic analysis. DNA and RNA can be isolated from CTCs, but only DNA is represented in the cfDNA extraction, and RNA is most commonly targeted from EVs

monitoring and characterization of cancer. Here we describe the most common sampling methods and their relative advantages and disadvantages for genomic profiling.

Formalin fixation and paraffin embedding

For a long time, FFPE has been used to preserve and solidify tumor biopsies for morphological examination [7]. While visually examining patient slides under the microscope, pathologists of the early 20th century could hardly have imagined the additional information locked inside the immobilized tissue sections before them. Fast-forward to today, the methods for fixation might not have changed much, but the methods for extracting and utilizing molecular information about a patient's cancer have advanced to the point of clinical significance.

FFPE has proven utility for morphological and immunohistochemical interrogation of cancerous cells; however, the use of FFPE poses several challenges to molecular characterization of genomic material [4]. Cell pellets and fresh frozen tissue routinely yield >10 μg DNA; however, in our experience with several thousand FFPE samples (as blocks, slides, or scrolls), they generally yield ≤1 μg DNA (unpublished data). Depending on the intended use of the genomic material, the amount of DNA yielded from FFPE samples might not be sufficient to produce high complexity sequencing libraries, which limits the sensitivity of variant calling. In addition to yield, the quality of extracted material can vary widely due to the interaction of formaldehyde with DNA. Several studies have reported both decreased yield and quality (measured by integrity and fragment length of extracted DNA) of FFPE-derived DNA with increasing length of storage [8, 9], though our experience is that even recently fixed samples can vary in quality across different submitting labs, suggesting that variation in processing protocols or reagents is a factor (unpublished data). Even seemingly good quality DNA extracted from FFPE samples can have higher variant false-positive rates compared to DNA from non-FFPE samples due to artifactual base changes resulting from formalin cross-linking of cytosine nucleotides [10].

In response to these issues, several methods have been developed to assess quality and quantity of extracted DNA (for example, using quantitative PCR to measure ratios of amplicons of increasing lengths), which can help to better triage incoming samples and, where appropriate, modify laboratory protocols (for example, by pooling of samples with similar quality scores together or using DNA repair enzymes prior to library construction) to maximize data utility [11–13]. Similarly, several sample preparation techniques have been developed to specifically process FFPE-derived (or otherwise degraded or low yielding) DNA samples, including some that leverage both DNA repair steps and alternative, more efficient adapter ligation strategies [14], while others have optimized automated library construction methods that use high-efficiency enzymes and have produced acceptable results for many FFPE samples [4]. Furthermore,

downstream variant-calling pipelines can detect some of the more common artifactual base changes through filtering [15], which highlights the need to capture and propagate sample type information to the analytical pipeline for optimal performance.

Generation of high quality genome sequencing data from FFPE-derived RNA is considerably more challenging than from FFPE-derived DNA. RNA extraction yields are generally higher than those of DNA (>10 μg; unpublished data), but FFPE-derived RNA is often highly degraded. Recently, methods for quality control of FFPE-derived RNA have been reported [16] and targeted selection methods have demonstrated utility in the generation of data to analyze transcriptomes and druggable fusions [17, 18].

As molecular profiling becomes more routine in clinical management, it remains to be seen if non-crosslinking tissue preservatives (for example, Optimal Cutting Temperature compound (OCT); PAXgene) might be used more frequently, considering that the improved quality of extracted nucleic acids can come at the expense of immunohistochemical performance [19].

Fresh frozen tissue and cells

Many of the integrity and yield issues associated with FFPE-derived material are avoided by the use of fresh frozen tissues and bulk cell pellets. Nonetheless, artifacts can still be introduced in the sample preparation process that are exacerbated by contaminating reactive elements in extraction buffers. Notably, high-energy acoustic shearing can mediate transversion artifacts through nucleic acid oxidation, which appear at low allele fractions [20]. This highlights how care must be taken at each step in the sequencing process, from nucleic acid extraction to sample preparation and detection, to avoid introduction of artifacts and biases that ultimately impact the sensitivity and specificity of clinical tests.

A specialized set of procedures is required to capture and sequence single cells. A common pre-analytical pipeline for single cell isolation is to disaggregate fresh tumor biopsy material followed by fluorescence activated cell sorting (FACS) prior to library preparation [21]. More efficient methods, such as micromanipulation (for example, circulating tumor cell enrichment and isolation from blood [22]), might be required for isolation of rare cells. Microfluidic isolation based on cell size has also been described [23].

Liquid biopsy

Genomic profiling from liquid biopsy is a rapidly growing area due to the relative ease of collection and lower associated costs. The total cost to obtain a surgical biopsy ranges from approximately $1000–4000 [24], whereas to obtain and extract nucleic acids from a liquid biopsy costs $100–200. Additionally, while tumor biopsy is the standard of care for primary diagnosis, tissue biopsies are not generally taken to monitor disease progression or to test metastatic lesions.

Multiple forms of liquid biopsy, such as cell-free DNA (cfDNA) [25], circulating tumor cells (CTCs) [26], and extracellular vesicles (EVs) [27], can be isolated from blood among other bodily fluids (see Fig. 1). Key considerations for molecular profiling of genetic information from lipid biopsies include special requirements for sample processing, low yield and purity of tumor-derived nucleic acids, and the uncertain false-negative rate.

Liquid biopsies are particularly sensitive to how they are handled, up to a certain point. For instance, blood must be properly collected (for example, into specialized blood collection tubes to minimize cellular DNA release [28]), stabilized, and fractionated within hours to days to mitigate degradation of cells or nucleic acids [29, 30]. Plasma fractionated from blood can be frozen for extraction of cfDNA or nucleic acids from EVs at a later date. For analysis of CTCs, positive selection (isolation of a target cell population by using an antibody that specifically binds that population) or negative depletion (depletion of all cell types except the cell type of interest) must be performed on the buffy coat (the fraction of an anticoagulated blood sample that contains most of the white blood cells and platelets following density gradient centrifugation) or whole blood prior to freezing an enriched cell pellet [31] (or single CTCs, if further purified [22]).

Liquid biopsies usually yield picogram to nanogram quantities of DNA or RNA, of which only a small fraction is derived from tumors [32]. In most individuals, peripheral blood mononuclear cells (PBMCs) and other non-tumor cells constitute the predominant source of cfDNA in blood [33]; similarly, methods to enrich for CTCs often result in significant carryover of PBMCs. In cancer patients, tumor purity in extracted cfDNA or enriched CTC samples is usually <5 % [32] and it is challenging to quantify tumor-derived EVs [22, 27]. If the total yield of nucleic acids is too low, whole genome amplification (WGA) or whole transcriptome amplification (WTA) might be required but can distort the original template [34]. Furthermore, the sensitivity to detect variants from low purity samples will be limited by the total yield or genome equivalents of cfDNA that are available for sequencing. Thus, the accurate profiling of tumor DNA or RNA in a sample that contains non-tumor DNA or RNA is challenging and requires specialized methods, such as error-correcting with molecular barcodes (tags of parsable (separable by software) sequence that are used to label individual starting molecules), also known as unique molecular indexes (UMI) [35], high efficiency library preparation kits for low input material [36, 37], or mutation enrichment [38]).

The false-negative rate in liquid biopsies is often difficult to determine. Tumor-derived cfDNA, EVs, or CTCs are sometimes undetectable in blood owing to technical or biological reasons. CTCs are not always enumerated prior to sequencing and might vary in quality of nucleic acids (for example, from apoptotic cells [39]) or might not express the surface markers used for identification. Similarly, detection methods for tumor-derived cfDNA or EVs often require probing for a select set of alterations and might not always include those present in a patient's cancer. Nucleosome positioning might also have an effect on the false-negative rate of sequencing cfDNA [33]. For these reasons, a negative result in a liquid biopsy assay might warrant follow-up testing from a tissue biopsy. Table 1 provides a summary of common pre-analytical issues, impacts, and contingencies associated with different sample types.

Matching the test to the intended use

Reduced costs in the generation of massively parallel sequence data and advances in wet lab and analytical techniques have resulted in a wide variety of options for tumor molecular profiling. Whole genome sequencing (WGS) [40], whole exome sequencing (WES) [4], large (300–600 gene) panels [3, 41, 42], small (<50 genes) panels [43], and hotspots (specific mutations in somatic genes) [44] have been used for somatic alteration profiling (Table 2). Selection of a specific genomic profiling test requires consideration of both pre-analytical (sample source) and analytical factors. One very important factor to consider is the intended use of the test.

Somatic variant calling from tumor genomic data is a complex and highly context-specific activity. Generally, variant sensitivity is a function of the depth of unique, high quality sequence reads at a site (read depth) and the proportion of molecules in the sample that are derived from the cancerous cells, known as the tumor allele fraction (AF) [45]. Tumor allele fraction is impacted by purity of the biopsy material, that is, how much "contamination" of normal DNA exists from non-cancer cells, and by the heterogeneity of the cancer itself. Tests that seek to assay known cancer driver genes or hotspots typically aim for high sensitivity to call these specific variants and are less concerned with novel or false positive incidental events. To achieve acceptable sensitivity (>99 %) for clinical use in solid tumor fresh frozen or FFPE samples, tests are typically run on samples with >20 % tumor purity (AF) and to high-read depths (>500× mean coverage) [3]. For liquid biopsies, these tests are commonly run at far greater read depths (>5000× mean coverage) and require use of molecular barcodes to achieve acceptable sensitivity and specificity for samples with low (<5 %) tumor purity [5, 35].

Achieving high mean read depths with broader capture methods such as WES or WGS is costly and inefficient if the clinically reported regions are limited to known hotspots or a selection of cancer driver genes; therefore, WES and WGS are less suited to routine diagnostic applications. Additionally, achieving a sequencing library with sufficient molecular complexity (number of unique molecules) to drive a whole exome or genome target to >500× coverage is challenging, particularly from FFPE-derived materials. Many diagnostic services sequence tumor material only, without matched normal germline data from the same patient (for example, whole blood). Analytically, this approach is more tractable if the area

Table 1 Common pre-analytical and sample preparation issues related to different sample types

Sample type	Common issues	Impact	Contingencies/solutions
Formalin-fixed, paraffin-embedded (FFPE)	• Low yield of DNA • DNA degradation • DNA base modification • RNA degradation	• Reduced complexity libraries; library failure; decreased sensitivity • Reduced complexity; library failure; decreased sensitivity • Increased false positive rate • Library failure; high duplication	• DNA repair; pooling of indexed libraries prior to capture (exomes or panels); specialized low input library methods • DNA repair; short amplicon amplification; specialized library methods • FFPE-aware filtering of variants; DNA repair • Selection-based or targeted preparation instead of polyA-based preparation
Fresh frozen tissue of bulk cells	• Buffer or process-induced modification of DNA bases	• Increased false positive rate	• Chelation of oxidative species; oxidation aware filtering
Single cells	• Low DNA yield • Whole genome amplification (WGA) bias • Low RNA yield	• Library failure • Increased false positives and false negatives • Library failure	• WGA • Optimized WGA • Whole transcriptome amplification (WTA)
Liquid biopsy	• Low DNA yield of cfDNA • Low purity of ctDNA in cfDNA • Low DNA yield from CTCs • Low RNA yield and quality from CTCs • Low RNA yield from EVs	• Library failure; reduced sensitivity • Reduced sensitivity • Library failure; reduced sensitivity; reduced specificity • Library failure • Library failure	• Optimized library preparation; specialized library preparation • High sequencing depth; molecular barcoding (UMIs) • WGA • WTA; specialized library preparation. • WTA; specialized library preparation.

Table 2 Common sequencing-based tests used in cancer genomics: their targeted regions, primary use cases, and limitations

Sequencing assay	Targeted regions	Primary use	Limitations
Whole genome sequencing	All genes, all exons, all non-coding regions	Discovery	Cost; depth; limited sensitivity for low allele fraction
Whole exome sequencing	All genes, all exons	Clinical research; panel-negative diagnostic testing; neo-epitope prediction	Cost; depth; moderate sensitivity for low allele fraction
Large gene panel	300–600 genes	Diagnostics; clinical trials; clinical research	Breadth; neo-epitope prediction
Small gene panel	<100 genes	Diagnostics; disease progression monitoring	Breadth; neo-epitope prediction
Hotspot panel	Portions of 50–80 genes, specific exons, variants	Diagnostics	Breadth; neo-epitope prediction
Transcriptome	mRNA	Variant validation; neo-epitope expression; fusion calling	Cost
Targeted RNA panel	Fusion genes	Fusion calling	Breadth; variant validation capability limited to targeted territory

being interrogated is smaller than a whole exome or genome.

However, in the immunotherapeutics field, WES might be a more appropriate test than a gene panel for the purposes of clinical management. Despite encouraging recent successes in immunotherapeutics (for example, the approval and use of checkpoint blockade inhibitors in a range of cancers), the understanding of predictors of response is incomplete [46]. Recent work has shown that mutational load and neoantigen load might be more useful biomarkers of response than specific driver gene mutations [47]. Similarly, the determination of mutational load and neoantigen expression is more predictive when whole exome data are used compared to large or small gene panels [48].

In cancer, WES is most commonly used in the clinical research setting, though diagnostic applications have been described [49]. One of the difficulties of WES for researchers is the so-called "long tail" of cancer genes, that is, the distribution of cancer-related genes with low frequencies in particular tumor types [50]. To address this phenomenon, research projects such as TCGA performed WES on a broad range of tumor types in an effort to better catalog the vast majority of these low prevalence cancer genes [2]. Recent efforts suggest that WES of liquid biopsies might be feasible to characterize metastatic and refractory tumors that would otherwise be challenging to biopsy [22, 51].

Single cell nucleic acid sequencing has been under development using many technologies. Single cell transcriptome profiling of tumor-derived cell populations is a highly sensitive and powerful tool for characterization of the tumor microenvironment and tumor heterogeneity [52]. Recent work by Tirosh et al. [21] highlights how this type of analysis could be leveraged in the future to profile tumors for likely development of drug resistance or candidacy for immune checkpoint blockade inhibitor treatment. Similarly, Miyamoto et al. [53] examined resistance development in prostate cancer using microfluidic enrichment of circulating tumor cells. Methods have been described for both RNA and DNA sequencing from single cells that leverage molecular biology techniques such as template-switching (Smart-seq) [54], incorporation of UMIs [55], and single nucleus sequencing [56]. Other methods have incorporated innovative technological platforms (nanodrops) to isolate cells and perform library construction at low cost, for example, Drop-seq [57] and the 10X genomics (Pleasanton, CA, USA) platform.

Bulk transcriptome sequencing and targeted RNA sequencing are now more widely adopted. Targeted RNA sequencing assays are used to capture and identify gene translocations in cancer samples [17]. Other sequence-based tests have been launched commercially that target common, potentially druggable oncogene fusions in *ALK*, *RET*, and *ROS1* in non-small cell lung cancer (NSCLC), a test historically carried out by immunohistochemical assays such as fluorescence in situ hybridization [58, 59]. Integrated analyses of exome (or genome) plus transcriptome profiles from a single tumor provide a more complete picture of the alteration landscape. Expression signatures from RNA can be used to determine if a driver gene candidate identified from DNA sequencing is actually expressed in the tumor or if resistance mutation expression levels change post-treatment [60].

Sequencing technology

Just as selection of the "test" is dictated by intended use, the choice of sequencing technology (or platform) is also an important consideration. Although there is less dimensionality in the sequencing landscape today, with Illumina (San Diego, CA, USA) capturing most of the application space, the complexity, scale, cost, and required throughput of the test are important factors in determining the optimal platform.

The required read length and generation of paired end reads are a primary consideration. Read length is an

important factor that relates to the type of genomic alteration events that might be queried and the overall accuracy of the placement of sequence reads relative to the target. In general, the most commonly used massively parallel sequencing platforms today generate short reads of a few hundred bases. This includes Illumina platforms (MiniSeq 2 × 150 bases, MiSeq 2 × 300 bases, NextSeq 2 × 150 bases, and HiSeq series 2 × 150 bases), also the Thermo (Waltham, MA, USA) Ion Torrent platform (Proton 1 × 200 bases), and the Qiagen (Hilden, Germany) GeneReader (100 bases). The utility of reads of this length is related to the type of assay being performed. For example, for amplicon sequencing (using "hotspot" panels), in general short read sequencing matches the size of the amplicon, and the amplicons can be designed such that the hotspot itself is located at a position where high quality can be expected (that is, not at the end of a read). Reads of a hundred or so bases are also useful for short variant detection using targeted sequencing of a gene panel or exome or in WGS. Similarly, for FFPE or cfDNA-derived materials, template lengths are generally shorter, so read lengths in the low hundreds of bases are appropriate.

Paired-end sequencing, which refers to sequencing a DNA fragment from both ends (the forward and reverse reads may or may not overlap), increases the utility of short reads in two ways. Some types of structural variation can be detected when the pairs of reads align to the genome in an unexpected way [61]. Sequencing both ends of fragments can also allow "de-duplication" in deep sequencing, where the occurrence of fragments with the exact same ends can be used to mask some reads as molecular duplicates, thus not adding to library complexity (for example, the MarkDuplicates tool in Picard [62]).

The main limitation of short reads (even if paired end) is in the discovery of fusion events or structural variation. Detection of known fusion events can be enabled by targeted assays that increase the utility of short reads by requiring mapping to a small or predefined event. Alternatively, specialized library construction methods to create long insert mate-paired libraries have shown some successes in structural variation detection [63]. For discovery of novel rearrangements, the most powerful approach involves long reads in which fusion or rearrangement events are spanned within the read. Options here include Pacific Bioscience (Menlo Park, CA, USA) instruments that generate reads of thousands of bases or the use of approaches such as the 10X Genomics platform, which links together short reads using a molecular barcoding approach. Another platform under active development in the long read space is the nanopore-based sequencing technology commercialized by Oxford Nanopore (Oxford, UK).

Ideally, the generation of very long reads would cost the same as an equal coverage of short reads, but this is not the case. Most dramatic decreases in sequencing cost have come from the platforms that generate short reads. For example, release of the Illumina HiSeqX decreased cost by threefold compared to the HiSeq2500: sequencing of a 30× human genome cost approximately $1500 on the HiSeqX compared to $5000 on the HiSeq2500. Sequencing the whole genome with long reads on a platform such as Pac Bio is cost prohibitive in most settings, at $20,000–80,000 per sample. In general, long read sequencing is used to sequence smaller (such as microbial) genomes or to target complex regions of the human genome (such as human leukocyte antigen genes) that are intractable for short read sequencing.

Short read sequencing costs vary considerably by platform, based on the instrument yield. For example, the lowest cost per Gb (billion bases) on a short read sequencer is approximately $15/Gb on the HiSeqX platform with an output of 1800 Gb bases per run. This level of throughput is appropriate for WGS which requires at least 100 Gb of data per sample, or considerably higher for tumor sequencing. Lower throughput platforms such as the MiSeq and HiSeq 2500 cost considerably more per Gb ($200/Gb and $45/Gb, respectively) but have an output per run (15 Gb for MiSeq, 1000–1500 Gb for HiSeq 2500) more appropriate for smaller scale sequencing, such as the panel test. A panel test of 100–200 genes might require 0.5–1 Gb per sample. Platform selection for this level of sequencing is a balancing act between the competing pressures of cost and turnaround time. To run most efficiently, multiple samples would be indexed, pooled, and sequenced on enough lanes to achieve the desired coverage. In practice, in the clinical testing world, the need for more rapid turnaround times necessitates running incomplete, and thus more expensive, batches. Technical features, such as template preparation techniques, sequencing chemistry, and error profiles are also important considerations. A review of technical differentiators is presented by Goodwin et al. [64].

Analytical considerations

Identification of somatic mutations of different types requires individually optimized approaches. There are many commonly used somatic variant callers each with varying performance attributes and optimizations [65]. In our own group, we are moving toward local realignment-based approaches for calling point mutations, insertions, and deletions (that is, Mutect 2, which utilizes the Haplotype Caller module of GATK [66] to call both single-nucleotide variants and indels). Fig. 2 provides an example of a best practice somatic calling workflow using GATK-Mutect. Considerations for single-

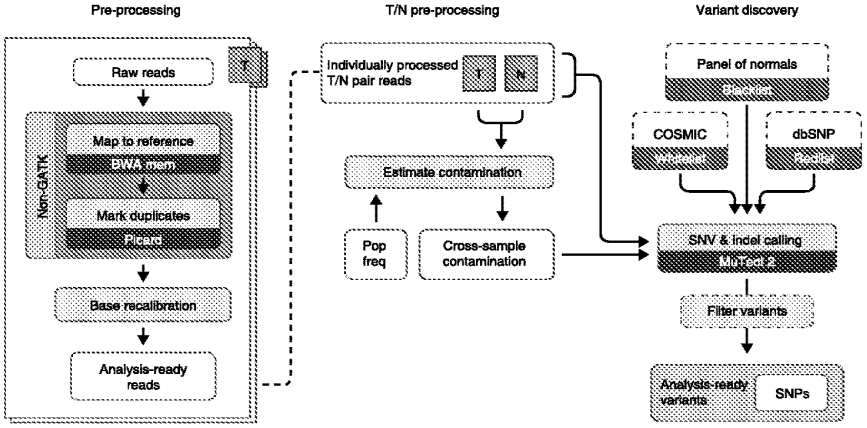


Fig. 2 Example of a best practices SNV calling workflow for somatic exome and genome data (reproduced with permission from [80]). Raw reads from the sequencing instrument are aligned and duplicate reads are marked (using the Picard tool). Vendor-assigned base quality scores are recalibrated for accuracy (based on position in read and other factors). Before running somatic analysis, both tumor and normal read groups are assessed for contamination, such as sample swap, cross-contamination, and tumor contamination in the normal sample. Somatic variants are those passing filter variants that are present in the tumor but not in the matched-normal sample. Several filters are used to control for technical noise in the system, which includes the variant allele frequency and a panel of normals (for more details see Cibulskis et al. [45])

nucleotide polymorphisms and InDel calling include depth of coverage and base quality scores. Base quality scores are often recalibrated from instrument-provided scores to account for context-specific and systematic variation in a process known as base quality score recalibration (BQSR). Somatic variant calling for very low allele fraction events, such as those in cfDNA, requires additional components. For example, these methods often use UMIs to enable more precise de-duplication and error correction of amplified libraries [35].

Structural variation (such as duplication, copy number variation (CNV), inversions, and translocations) has traditionally been difficult to call with standard short read data. WGS is the most well characterized data type for structural variation calling, particularly when supplemented by long linking information or long reads. Recent technological advances that use droplet partitions (emulsions) and unique molecular barcodes have made this data type more tractable [67].

Some methods for variant calling rely on having a matched normal sample from the same patient to filter individual germline variants, which would otherwise be considered false-positive somatic calls. Additionally, a set of data created with non-cancer samples that uses the exact same assay and sequencing technology, a so-called "panel of normals" (PoN), is useful for removing artifacts due to systematic process variation in the library preparation or sequence generation steps [45]. Specific PoNs are needed for each different process type, for example, cfDNA low input library construction requires its own PoN for filtration. Some groups do not use matched normal material. In order to minimize false-positive calls, these groups either focus on calling previously characterized driver events in known oncogenes (in the case of hotspot panels), or use advanced filtering methods—unmatched normal, PoN, large germline databases (for example, 1000 Genomes, ExAc)—to remove non-somatic variants [48]. Specificity can be further increased by review of candidate mutations by an experienced molecular pathologist and cross-referencing somatic mutation databases such as COSMIC for pathogenicity information [48].

An area of particular interest at present is immunoinformatics, which refers to the analysis of patient genomics data to profile their immune system, and in the case of cancer patients, the tumor microenvironment, with the aim of identifying biomarkers of response to immune blockade inhibitors [47]. Software tools now exist that use patient exome and transcriptome data to call HLA types and predict T- and B-cell epitopes. For a review of these methods, see Backert and Kohlbacher [68]. T-cell receptor (TCR) profiling through targeted amplification and sequencing of the CDR3 region is another application that has seen adoption for both diagnostics [69] and clinical research [70].

Accurate analysis of CTC single-cell data is confounded by the errors imparted by the WGA process. WGA introduces allelic distortion and polymerase errors that result in exceedingly high false-negative and false-positive rates, in contrast to bulk sequencing, and affect our ability to confidently detect all classes of genomic alterations [34]. Strategies to overcome the error modes of WGA include joint analysis together with bulk sequencing of matched tumor tissue or other independently

amplified single cells [22, 71]. These methods are reviewed by Gawad et al. [72].

So far, we have discussed only the technical aspects of analysis to identify somatic variation in the patient's tumor. Depending on the size of the territory interrogated, the number of somatic variants found can range from a few (in a hotspot panel) to a few hundred (in a whole exome). The next step in the process prior to clinical decision-making is the annotation of variants with functional information and interpretation of the likely impact of the events in the context of the patient's disease. For germline diseases, molecular geneticists routinely use large population variant frequency databases, such as ExAc [73], to filter out events previously found in the population. These same resources can be used to filter germline events from somatic variation [48] but are not useful for annotation or filtration of actual somatic events. To annotate and filter somatic events, a large database of somatic variation, COSMIC, is often used [74] and, increasingly more clinically curated databases such as ClinVar [75] are used to query the pathogenicity of specific variants. Unfortunately, a lot of deep knowledge about specific tumor type variation still resides in proprietary databases maintained by commercial diagnostic companies, though efforts are underway to free or recreate these datasets and others as publically available resources [76–78]. Finally, given the complexity of the data types and the number of variables that can impact the results, there is still a need for expert human review in the field of clinical genomics. Typical activities for molecular geneticists, pathologists, and in some cases molecular tumor boards (comprising specialists who discuss the results of advanced genomic diagnostic tests of cancer patients), range from variant review and visualization, using tools such as the Integrated Genome Viewer (IGV) [79], to prioritization of variants based on clinical or professional experience and the context of the patient's disease.

Conclusions

Never before in the history of molecular oncologic pathology have we had the ability to examine a patient's tumor with the resolution or richness of information that it is possible to generate today. With this increased resolution comes a lot of additional considerations. In order for genomic information to be useful in a clinical setting we need the data produced to be accurate, actionable, and timely. Advances in sequencing technologies have made the sequence data itself extremely accurate in most contexts, such that the major sources of false positives and false negatives today are caused by pre-analytical factors (such as chemical or physical damage of DNA/RNA, limited material, or inappropriate handling) and post-analytical factors such as variant calling limitations. Upfront consideration of intended use of genomic data and careful selection of both assay type (exome, transcriptome, targeted panel) and bioinformatic analysis methodology are required for optimal utility. Future advances in solid tumor clinical research will likely see more integrated analyses of a tumor. That is, not just a targeted gene panel test, but a targeted panel, plus a targeted fusion test, plus an immune cell profile. A more expansive profiling, which offers the ability to cross-validate findings and gain a more complete molecular picture of a tumor, could incorporate a deep whole genome (with linked reads for SV detection) plus a transcriptome (for expression, fusions, and variant validation) plus an epigenetic test (for dysregulation). The methods for such testing exist today but require continued optimization to work with available sample types and amounts and more integrated analytical platforms to bring the multi-omic datasets together in a meaningful and practically interpretable way.

Liquid biopsy represents an exciting new class of sample matrix that enables more frequent and facile monitoring of tumor burden and could allow for more rapid treatment course correction. Further advances in liquid biopsy methodology could enable not just post-diagnostic sampling but also pre-diagnostic screening for cancer risk, as has been shown with the application of cfDNA in the non-invasive prenatal testing (NIPT) field. With continued technological advances and increasing availability of variant databases for annotation and interpretation, the use of genomic testing in clinical cancer management seems likely to continue to progress toward standard of care, though non-trivial issues such as access to testing, wide-spread physician education, and adoption of testing, and reimbursement for testing will likely be the rate limiting steps.

Abbreviations

AF: Allele fraction; cfDNA: Cell-free DNA; CNV: Copy number variation; CTC: Circulating tumor cell; ctDNA: Circulating tumor DNA; EV: Extracellular vesicle; FFPE: Formalin-fixed paraffin-embedded; NIPT: Non-invasive prenatal testing; PBMC: Peripheral blood mononuclear cell; SNP: Single-nucleotide polymorphism; SNV: Single-nucleotide variants; SV: Structural variation; TCGA: The Cancer Genome Atlas; UMI: Unique molecular index; WES: Whole exome sequencing; WGA: Whole genome amplification; WGS: Whole genome sequencing; WTA: Whole transcriptome amplification

Acknowledgements

The authors wish to thank Leslie Gaffney and Lior Friedman for their help with Fig. 1.

Funding

Not applicable.

Availability of data and materials

Not applicable.

Authors' contributions

NL, VA, and SG all contributed to writing the manuscript. All authors read and approved the final manuscript.

Competing interests

NL has participated in a Key Opinions Leader panel at New England Biolabs. The authors declare that they have no other competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Published online: 26 October 2016

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MINIREVIEW

Different Facets of Copy Number Changes: Permanent, Transient, and Adaptive

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Chromosomal copy number changes are frequently associated with harmful consequences and are thought of as an underlying mechanism for the development of diseases. However, changes in copy number are observed during development and occur during normal biological processes. In this review, we highlight the causes and consequences of copy number changes in normal physiologic processes as well as cover their associations with cancer and acquired drug resistance. We discuss the permanent and transient nature of copy number gains and relate these observations to a new mechanism driving transient site-specific copy gains (TSSGs). Finally, we discuss implications of TSSGs in generating intratumoral heterogeneity and tumor evolution and how TSSGs can influence the therapeutic response in cancer.

It was long thought that the DNA sequences of healthy individuals were 99.9% identical to each other (1). However, genome-wide sequencing efforts in individuals from multiple ethnicities have revealed more variations in the genetic architecture than were previously appreciated (2–4).

These genomic alterations have been termed structural variants, which are further classified as microscopic or submicroscopic, depending on the amount of DNA involved (5). The microscopic variations have historically been identified through chromosome banding techniques (6) and comprise at least 500 kb of DNA (7). Examples of these variants are whole-chromosome gain or loss (referred to as aneuploidy [7, 8]), translocation (change in location of a chromosomal segment [9]), deletion (deletion of a DNA segment relative to the rest of the chromosome [10]), duplication (a chromosomal segment occurs in two or more copies per haploid genome [11]), and inversion (reversal in orientation of a DNA segment compared to the rest of the chromosome [12, 13]). A schematic of structural variants resulting in copy number changes is shown in Fig. 1. With the development of more sophisticated tools, such as array-based comparative genomic hybridization (GGH) arrays (14–16), smaller variants (submicroscopic alterations) in the size range of 1 to 500 kb can be detected (5). Genome sequencing has further revealed small insertions and deletions (indels) spanning from 1 to 10,000 bp across the human genome which could cause considerable variability in the human population (17, 18).

The most common variant identified under submicroscopic alterations is copy number variation (CNV). CNV is defined as a genomic segment of more than 1 kb present at a variable copy number in comparison to a reference genome (19–22). The first studies documenting the genome-wide presence of CNVs in the normal human genome came from work in the laboratories of Lee (23) and Wigler (24). These studies described more than 200 large-scale CNVs (LCVs; about 100 kb or greater) in normal individuals. These studies also paved the way for the creation of the Database of Genomic Variants (DGV) in 2004, which catalogs all the human CNVs and structural variations present in healthy individuals.

The sequencing efforts from the International HapMap Consortium (25) and 1000 Genomes Project (26) have led to the identification and frequency determinations of novel CNVs in the human genome. CNVs are now known to contribute to 4.8% to 9.5% of the variability in the human genome (27, 28), which is more than what is accounted for by single nucleotide polymorphisms (SNPs; accounting for 0.1% of the variations) (29). Recently, the CNV map for the human genome was constructed (28), and it documented all the small- and large-scale CNVs present in normal healthy individuals. CNVs can either have no phenotypic consequences in individuals (4, 23, 24) or lead to adaptive benefits that have been observed in a wide range of species (5).

One of the major challenges in the field is to distinguish benign CNVs (events that do not lead to phenotypic consequences) from pathogenic CNVs that underlie diseases (30). Pathogenic CNVs are often associated with deleterious consequences because of an imbalance in gene dosage (31) and/or aberrant chromosomal structure (5, 7, 32, 33). Pathogenic CNVs have been associated with several disorders, including the following: obesity (34), diabetes (35), developmental disorders (36), psychiatric diseases (37) such as autism spectrum disorder (38), schizophrenia (39), and Alzheimer's disease (40, 41), and cancer (42–44). In this review, we focus mainly on copy number alterations observed in cancer and their functional implications.

CNVs can either be present in the germ line or can arise in phenotypically normal tissues and organs, which are referred to as somatic CNVs (45, 46). Instead of being randomly present in the genome, CNVs are preferentially found to occur in regions that are rich in low-copy-number repeats (segmental duplications) (47–50), heterochromatic areas (e.g., telomeres and centromeres), and replication origins and palindromic regions (28). There are several proposed mechanisms that underlie the generation of somatic CNVs: nonallelic homologous recombination (NAHR), nonhomologous end joining (NHEJ), defects in DNA

Accepted manuscript posted online 11 January 2016

Citation Mishra S, Whetstine JR. 2016. Different facets of copy number changes: permanent, transient, and adaptive. Mol Cell Biol 36:1050–1063. doi:10.1128/MCB.00652-15.

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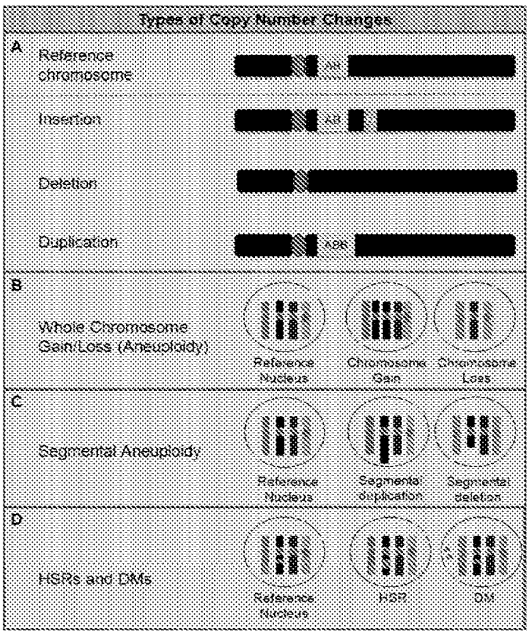


FIG 1 Types of copy number changes. (A) Representative examples of structural chromosomal alterations are shown, with a new sequence insertion (D), deletion of region AB, and duplication of sequence B (ABB). The reference chromosome is shown at the top. (B) Aneuploidy with whole chromosome gain (the extra black chromosome) and loss (of black chromosome) are depicted with respect to a normal mitotic reference nucleus. (C) A part of a chromosome (black) can be amplified or deleted (black), giving rise to segmental aneuploidy. This is demonstrated here as involving rearrangement of only one chromosome. A more likely scenario is an unbalanced translocation, which is not shown in the figure. (D) Homogenously staining regions (HSR) and double minutes (DMs) are chromosomal structures that are generated as a consequence of gene amplification. HSRs are repeated units clustered at a single chromosomal locus (red), and DMs are unstable circular extrachromosomal DNA structures lacking a centromere or a telomere. In addition to these structures, amplicons can be present at a number of loci in the genome (not shown).

replication, and DNA damage response and repair pathways. These mechanisms have been extensively discussed elsewhere; therefore, we refer our readers to several reviews (32, 33, 51).

In this review, we explore the relationship between copy number changes and biological consequences, with a particular focus on development and tissue homeostasis under physiological as well as pathological conditions. This review focuses on these relationships, especially in the context of cancer. We further discuss a recently discovered process driving transient site-specific copy number gains (TSSGs) in cancer cells and its implications during adaptive responses such as stress and chemotherapeutic sensitivity.

COPY NUMBER CHANGES IN DEVELOPMENT AND PHYSIOLOGY

Chromosomal copy number changes and the associated gene amplifications and losses are observed during development in both lower and higher eukaryotes [reviewed in reference 7]. The appearance of CNVs during normal biology suggests that copy number changes can have important functional consequences. A common hypothesis is that increased gene dosages during development provide an advantage during selective pressures and environmental conditions (7). Here, we discuss examples from developmental biology and their relationships to functional impact. We also highlight the relationship between somatic CNVs and tissue homeostasis.

Several lower and higher eukaryotes use gene amplification to respond to cellular signals (Fig. 2). Electron microscopy studies in the early 1970s demonstrated that ribosomal genes are amplified for the production of large amounts of ribosomes required during early embryogenesis (52). Ribosomal DNA (rDNA) amplifications were observed during oocyte formation in amphibians such as *Xenopus leavis* (53–55), insects such as water beetles (56), molluscs (55), and in the macronuclear rDNA of *Paramecium* (57) and *Tetrahymena* (58). Thus, such an increase in rDNA synthesis to meet higher protein synthesis demands in different tissues highlights gene amplification as a common principle in developmental biology.

Besides rDNA, specific chromosomal regions identified as "DNA puffs" are amplified and expressed to form structural proteins required for cocoon formation in the salivary gland of sciarid flies (59, 60). Amplification of the DNA puffs occurs in response to the hormone ecdysone, which is required during larval development (60). Another example of gene amplification triggered by developmental signals can be observed during eggshell formation in *Drosophila melanogaster* (61). Eggshells require amplification of chorion genes in the follicle cells of the ovary, and these genes are expressed late in differentiation (61, 62). The amplifications of only specific chromosomal regions and genes and not the whole genome highlight the specific response that can occur across organisms. These examples suggest the ability of cellular cues to trigger these site-specific amplifications, which raises the question about what molecular mechanisms underpin this selective amplification across species.

Examples of copy number variations have been reported in various tissues in mammals. Using techniques such as spectral karyotyping (SKY), fluorescence *in situ* hybdridization (FISH), and single-cell sequencing approaches, various groups have reported both small- and large-scale changes in chromosomal copy numbers in mouse and human tissues, particularly in neurons, liver cells, and skin fibroblasts (Fig. 2). For example, approximately 33% of the neuroblasts in the embryonic mouse brain and 20% of neurons in the adult mouse cerebral cortex showed aneuploidy (63). The reduction in aneuploidy in the adult brain was hypothesized to be due to a neuroblast programmed cell death mechanism during brain development (64). Westra and colleagues also uncovered that 15 to 20% of neural progenitor cells in both mouse and human cerebella exhibited aneuploidy (65) (Fig. 2).

Additionally, high levels of subchromosomal CNVs (deletion and duplication events) were observed in the human frontal cortex neurons. Multiple copy number changes were noted within a small set of neurons, suggesting that CNVs might be restricted to either individual cells or specific neural lineages (66). These data suggest that the generation of copy number changes is an important process for achieving diversity in the neuronal populations during central nervous system development. However, this possibility has yet to be proven. It was reported that the transcripts arising from CNVs in the mouse brain are more tightly regulated than are other tissues such as lung, liver, heart, kidney, and testis (67). It would be important to determine the rate of correlation

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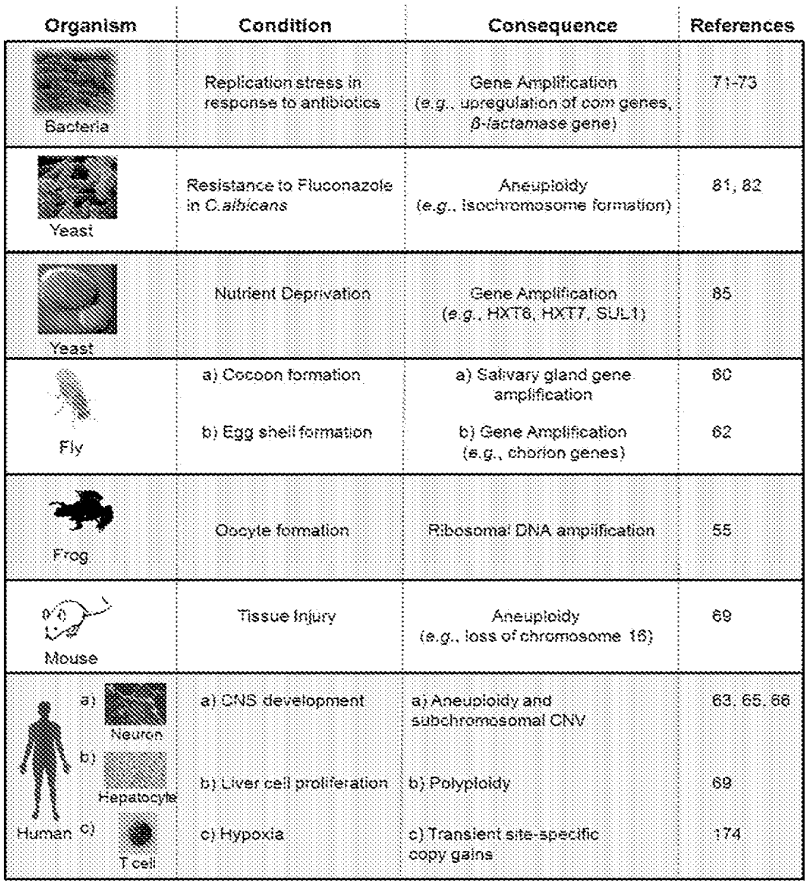


FIG 2 Copy number changes during normal development and physiological conditions. Representative copy number changes are shown for organisms and specific tissues under different developmental and physiological conditions. Please refer to text for detailed descriptions and corresponding references.

between CNVs and expression changes in the human brain and whether there are underlying functional consequences of the affected transcripts in generating neural diversity and plasticity.

Somatic CNVs are also observed in mammalian hepatocytes and skin. A study by Duncan and colleagues suggested that approximately 50% of normal adult hepatocytes have changes in chromosomal numbers (gains or losses) such that genetically diverse sets of cells are present in the liver (68, 69). However, single-cell next-generation sequencing has reported a lower level of aneuploidy (<5%) in cells of liver, skin, and human neurons (70). The differences in the reported levels of aneuploidies could reflect the different types of assays employed to follow copy number changes (i.e., FISH and SKY versus single-cell sequencing, respectively).

The genetic variation resulting from the changes in copy number could be a mechanism employed during tissue development in order to achieve diversity in cell populations. Copy number variations may allow developing tissues to adapt to cellular and growth requirements during tissue expansion and organ development. Another advantage for the observed CNVs could be to adapt to encountered metabolic or toxic challenges, especially by hepatocytes (see the discussion in "Mammals," below). By identifying the regulatory features for regions undergoing CNV and the affected genes in different tissues, we would be able to understand tissue-specific gene expression and underlying diversity within tissues.

COPY NUMBER CHANGES AS AN ADAPTIVE RESPONSE

Many studies in bacteria, yeast, and mammals have shown that copy number changes can arise as a consequence of selection, which may allow cells to exhibit an increased fitness and/or survival advantage. In this section, we discuss the relationship between different cellular conditions and the emergence of CNVs from different species (Fig. 2).

Bacteria. Acquisition of antibiotic resistance can occur through the uptake of foreign DNA harboring resistance genes through the bacterial competence pathway (71). A recent study by Slager et al. demonstrated that different species of bacteria could increase the copy number of genes involved in the competence pathway (*com* genes) in response to antibiotics causing replication stress (72). These genes are located closer to the origin of replication (OriC), and their amplification occurs through multiple origin firing events at the OriC, which increases their copy number and transcription rates. In *Salmonella enterica* serovar Typhimurium, gene amplification aids in the development of antibiotic resistance. Adaptation to the antibiotic cephalosporin occurred through ampli-

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fication and increased gene dosage/expression of the β-lactamase gene (bla_{TEM-1} [73]). The enzyme β-lactamase results in the hydrolysis of cephalosporin (74, 75), which results in a reduced drug response.

These highlighted examples illustrate the impact selective pressure can have on DNA amplification and gene expression in bacteria (Fig. 2). Additional examples have been observed and are discussed in a review by Sandegren et al. (76). Taken together, the existing data illustrate the relationship between input signals and changes at distinct regions of the bacterial genome. In the future, it will be interesting to know if this selection is based on fitness or the result of targeted DNA replication in prokaryotes.

Yeast. Similar to bacteria, yeasts also exhibit changes in DNA content based on selective pressure. For example, gene rearrangements and copy number changes have been observed in *Candida albicans* when it is passaged through a murine host (77). It has been hypothesized that these changes in ploidy could generate the genetic and phenotypic diversity required for adaptation in the new host environment. Consistent with these observations, CNV has been associated with antifungal drug resistance and adaptive benefits (78, 79). For example, fluconazole treatment for *C. albicans* infection results in the development of whole-chromosome gains and aneuploidy (80). Upon CGH analyses for the copy number changes in 70 azole-resistant and -sensitive strains, Selmecki et al. found increased levels of aneuploidy in resistant strains (50%) compared to the sensitive ones (7.14%) (81, 82). Trisomies of chromosome 5, including a segmental aneuploidy consisting of an isochromosome (formed by the attachment of two left arms of chromosome 5 around a single centromere), were also associated with azole resistance. Gains of this isochromosome were associated with increased expression of genes involved in drug resistance (82). Some of these genes encoded efflux pump proteins involved in resistance: an ATP-binding cassette (ABC) transporter and a multidrug resistance transporter (83). Other genes were *ERG11* (a target of fluconazole [84]) and *TAC1* (a transcription factor that upregulates ABC gene expression [82]). There is a need to identify other structural variations and affected genes conferring a survival/adaptive advantage against antibiotics and whether these changes are conserved across other fungal species.

Consistent with gene amplification conferring a selective advantage, *Saccharomyces cerevisiae* cells exposed to nutrient deprivation exhibited gene amplifications that provided a cellular benefit (85). For example, glucose limitation in cultures resulted in the amplification of genes encoding glucose transporters (*HXT6* and *HXT7*), while sulfate limitation resulted in the amplification of *SUL1*, a gene that encodes a high-affinity sulfate transporter (Fig. 2). The question remains as to whether these physiological input signals are able to drive selective DNA gains through a hardwired mechanism, as observed in mammalian cells (discussed in "TSSGs, Tumor Heterogeneity, and Cancer Evolution," below), or are the result of random selection. Resolution of this issue could have a profound impact on our understanding of cellular fitness and responses to antibiotics.

Mammals. Mammals are no exception to selective pressures promoting copy number changes or copy number alterations that impact biological consequences. For example, the copy number of the human salivary amylase gene *AMY1*, which encodes an enzyme that aids in the hydrolysis of starch, is increased in populations that have a higher starch content in their diets compared to low-starch-consuming populations (86). The increased copy number of *AMY1* also correlated with increased salivary amylase protein levels. This illustrates how diet-induced selective pressures could influence copy number polymorphisms in mammals. Other examples and the role of copy number polymorphisms in human adaptation have been reviewed elsewhere (33, 87, 88). While these studies are correlative and suggest that the environment impacts selection, they have yet to be shown to be causal.

Increased or decreased copy numbers of certain genes can predispose an individual to diseases. For example, susceptibility of individuals to HIV/AIDS infection is increased in populations with a decreased copy number of the chemokine gene *CCL3L1*. This chemokine serves as a ligand for HIV coreceptor CCR5, which inhibits viral entry by binding to CCR5. However, HIV-resistant individuals show duplications of the CCL3L1 locus (17q21.1) and increased CCL3L1 copies imparting resistance to HIV infection (89). Other examples of CNVs promoting susceptibility to diseases can be found with psoriasis (associated with a copy number gain of the β-*defensin* gene [90, 91]), pancreatitis (a copy number gain of *PRSS1* [92]), and Crohn's disease (a copy number loss of *HBD-2* [93]), among others (20, 94). The question remains as to whether there are mechanisms that would allow such changes to occur immediately in response to stimuli in the population or whether they reflect some mutation that was selected over time.

Somatic mosaicism for CNVs within tissues can provide an adaptive response as well. CNVs within the liver can provide protection against tissue injury. Duncan et al. demonstrated in a chronic liver injury model that selective gene loss could provide resistance to liver injury (95). Deficiency of fumaryl acetoacetate hydrolase (encoded by *FAH*; the enzyme is required in tyrosine catabolism) causes a buildup of fatty acids and toxic metabolites that result in liver failure, known as tyrosinemia. Conversely, deletion of the genes encoding enzymes that function upstream of *FAH* (e.g., homogentisic acid dioxygenase [HGD]) is found to be protective for tyrosinemia. Mice deficient for *FAH* and heterozygous for a mutation in *HGD* can generate healthy normal hepatocytes. These injury-resistant, aneuploid hepatocytes (characterized by the loss of chromosome 16) are present in the liver and undergo expansion only when the liver is exposed to injury, demonstrating an adaptive response of cells to metabolic or toxic challenges.

Taken together, these few examples illustrate the CNVs present within populations and individual tissues and how these are associated with phenotypes. These data also emphasize the variations in the genome and how the environment and selective pressures can impact genetics. However, the question remains as to whether these genetic events occur after random selection or are the result of unidentified mechanisms that selectively alter the genetic landscape in response to external stimuli and, in turn, drive targeted *de novo* genetic changes.

COPY NUMBER ALTERATIONS IN CANCER AND THEIR IMPLICATIONS IN ACQUIRED DRUG RESISTANCE

Copy number alterations involving whole chromosomes and/or specific chromosomal segments are frequently observed in cancer (96, 97). Gains/amplifications of oncogenes and loss/deletion of tumor suppressor genes have been historically found to be major drivers of tumor development. For example, amplifications of *EGFR* in gliomas (98), *MYCN* in neuroblastoma (99), *MYC* in acute myeloid leukemia (100), and *ERBB2* in breast (101), ovarian

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(102), and lung cancers (103) have been reported. Similarly, loss/deletions in tumor suppressor genes such as *PTEN* (104), *TP53* (105), and *VHL* (106) have been observed in a variety of tumors. The dependence of tumors on specific oncogenes for their proliferation and survival is referred to as oncogene addiction (107). By targeting these oncogenes, tumor cell growth becomes limiting or abrogated. For example, clinical success has been observed with the ERBB2 antibody trastuzumab (Herceptin) in the treatment of *ERBB2*-amplified breast cancer (108), crizotinib in the treatment of *MET*-amplified non-small cell lung cancer (109), and the epidermal growth factor receptor (EGFR) inhibitors gefitinib and erlotinib (which blocks the catalytic activity of EGFR) in lung cancer patients with *EGFR* mutations (110).

In addition to oncogene amplifications, copy number alterations of different chromosomal regions have been observed in cancer. A genome-wide analysis of copy number alterations in cancer demonstrated a total of 76,000 gains and 55,000 losses across the 3,131 cancer samples analyzed (96). A typical tumor type is comprised of 17% amplifications and 16% deletions, compared to less than 0.5% in normal samples (96). These data suggest that somatic copy number alterations are a frequent feature in cancer cells. Analyses across 17 tumor types demonstrated that 25% of the genome is affected by whole chromosome alterations and 10% of the genome by short chromosomal changes (focal events) in a typical tumor (96). Interestingly, the focally amplified regions often harbor known oncogenes (e.g., *MYC*, *CCND1*, *EGFR*, *NKX2-1*, and *KRAS*), while the focally deleted genomic loci contain tumor suppressor genes (*TP53*, *CDKN2A/B*, and *Rb1*). These observations suggest that the selective pressures associated with tumorigenesis might influence targeted amplification or deletion of specific regions within tumor cells instead of occurring randomly, which would be reminiscent of the observations seen in bacteria and yeasts (Fig. 2).

Focal amplifications can also harbor oncogenes or prosurvival genes that can influence drug responses. For example, ~10% of cancers have a focal amplification of chromosome 1q21.2 that contains the antiapoptotic gene *MCL1* (96). Another focally amplified antiapoptotic gene that is observed in cancer is *BCL2L1* on chromosome 20q11.21 (96). Both of these genes are important for cell survival; hence, their amplification within tumors could confer a distinct survival advantage. Consistent with this notion, Beroukhim et al. demonstrated that increased expression of these genes protected tumor cells from chemotherapy (96).

Chromosomal alterations in several distinct regions also influence pathogenesis in different tumor types. For example, in multiple myeloma (MM), disease progression is characterized partly by the focal amplifications of a proximal region of chromosome 1q (chr 1q). Several studies have identified a region of 10 to 15 Mb that corresponds to a chr 1q12-23 amplicon in MM. This region contains a large number of genes with amplifications or deregulated expression involved in myeloma pathogenesis, including *CKS1B* (111, 112), *MUC1* (113), *MCL1* (114), *PDZK1* (115), *IL-6R* (116), *BCL9* (117), and *UBE2Q1* (118). The amplification of a drug-resistant oncogene, *CKS1B*, and the proximal chr 1q21 region has been reported in ~40% of newly diagnosed MM cases and in 70% of patients with tumor relapse (119, 120). The gains observed in *CKS1B* are in the range of one to three copies (111, 112). These focal amplifications are associated with poor prognosis and reduced response to cisplatin therapy (111) (Table 1). Studies in cell cultures have further demonstrated that overexpression of *CKS1B* confers a reduced response to cancer chemotherapeutics (121). Similarly, amplification of the *PDZK1* gene within the chr 1q12-q22 region has been observed in primary cases of MM, and the overexpression of *PDZK1* in cells conferred resistance to melphalan-, vincristine-, and cisplatin-induced cell deaths (115) (Table 1).

TABLE 1 Partial list of amplified genes that impact drug resistance[a]

Cancer type	Therapeutic agent(s)	Gene(s) implicated in resistance (reference[s])
Multiple myeloma	Bortezomib, cisplatin	*CKS1B* (111, 121, 126)
	Melphalan, cisplatin, vincristine	*PDZK1* (115)
	Dexamethasone	*FGFR3* (127)
Ovarian	Cisplatin, CDK2 inhibitors	*CCNE1* (128, 142)
	Paclitaxel	*MDR1* (129, 130)
Lung	Gefitinib	*MET* (123, 125)
	Paclitaxel	*MDR1* (129, 130, 131)
	Crizotinib	*ALK*, *KIT* (132)
Breast	Trastuzumab	*MET* (133), *IQGAP1* (134)
Colorectal	Gefitinib	*MET* (124)
	5-Fluorouracil	*TMYS* (135)
CML	Imatinib	*BCR-ABL* (136)
Melanoma	Vemurafinib	*BRAF* (137, 138), *BCL2A1* (139)
Leukemia	Methotrexate	*DHFR* (140, 141)

[a] We apologize for not being able to cite or include all studies related to gene amplification and drug resistance.

Gene amplifications are associated with drug resistance in several tumors (122–141) (Table 1). For example, ovarian cancer patients with a chr 1q12-21 amplification are more resistant to cisplatin treatment (142, 143). Amplifications of cyclin E1 (CCNE1) are present in 25% of high-grade serous ovarian cancers and are associated with poor survival and impart resistance to CDK2 inhibitors (144) (Table 1). In the case of non-small cell lung cancer cells, an 11- to 13-fold-higher copy number of chr 7q21.12 was detected by CGH in an acquired paclitaxel-resistant lung cancer model (study NCI-H460/PTX250) compared with the parental cell line (study NCI-H460). Most of the genes within this region were also highly expressed, including a multidrug transporter gene, *MDR1/ABCB1* (131). These examples highlight how distinct regions in the genome are focally amplified and relate to altered patient outcome and cancer cell drug responses. Whether selective chromosomal alterations and gene amplifications in cells result from a stochastic process or occur in a directed manner in consequence to therapeutic pressure is yet to be determined.

DNA AMPLIFICATION AND CANCER CHEMOTHERAPEUTIC RESISTANCE

Gene amplification serves as a biochemical basis for drug resistance in mammalian cells. This relationship to resistance was first documented in seminal work by Hakala (145–147) and Fischer (148) in the 1950s. They isolated highly resistant tumor cells under the presence of increasing concentrations of the drug methotrexate (MTX). MTX competitively inhibits the enzyme dihydrofolate reductase (DHFR), which catalyzes the conversion of dihydrofolate to active tetrahydrofolate, which is required for the *de novo*

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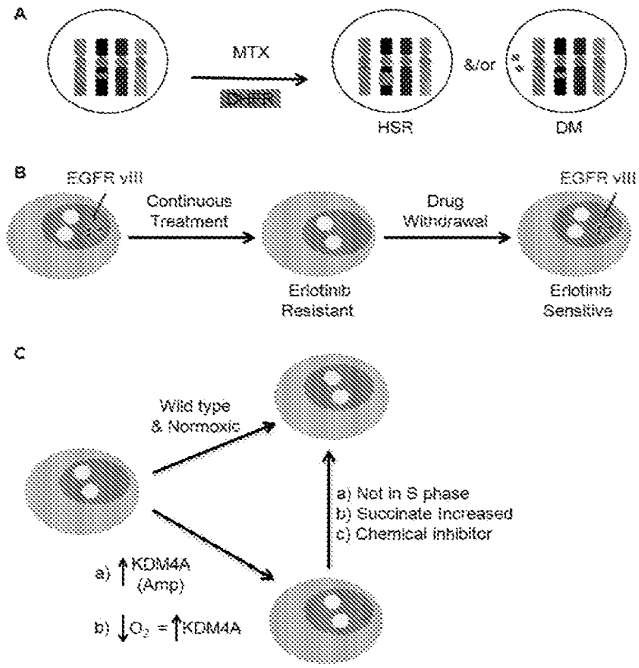


FIG 3 Permanent and transient adaptive changes under different cellular conditions. (A) Methotrexate treatment results in the amplification of the *DHFR* gene (shown in red). DHFR can persist either as a stable structure, such as an HSR, or as an unstable DM that is lost upon subsequent cell division. (B) Continued treatment of glioblastoma cells with a tyrosine kinase inhibitor such as erlotinib results in the loss of EGFR vIII-positive extrachromosomal DNA (red) and its reemergence upon drug removal. (C) Hypoxia or overexpression of histone demethylase KDM4A results in site-specific genome amplification (purple), which is generated every S phase. The amplification is reversible after KDM4 inhibitor treatment or with increased succinate dose. Studies related to these data are discussed in the text.

synthesis of thymidine. They found that the drug-resistant cells had around 155 times the level of DHFR. They also found that the drug-resistant phenotype was unstable in murine sarcoma 180 cells, which coincided with the reduced DHFR enzymatic activity. Schimke's laboratory further characterized the mechanistic basis for the increased DHFR levels (149). It was shown that the cells developed resistance to MTX by overproduction of DHFR protein as a result of selective gene amplification (150). It was from the work of the Biedler and Spengler (151, 152) and Schimke (150, 153) laboratories in the 1970s that the presence of cytogenetic structures associated with MTX-resistant cells was demonstrated. They found that gene amplification accounts for the overproduction of DHFR in stable and unstable drug-resistant cells (Fig. 3A).

Gene amplification forms two common structures: extrachromosomal double minutes (DMs) and intrachromosomal homogenously staining regions (HSRs). DMs were first observed in lung cancer cells in 1962 (154). They are defined as chromatin bodies that lack centromeres and telomeres that are not transmitted to 100% of daughter cells during mitosis (155) (Fig. 1D). HSRs are chromosomal structures containing permanently integrated genes (Fig. 1D). These were first described by Biedler and Spengler in 1976 (152) in drug-resistant cells. DHFR was found to reside on HSRs in highly methotrexate-resistant CHO cells (156) and murine leukemia cells (157). Kauffmann et al. further showed that the amplified *DHFR* genes were associated with DMs in unstable MTX-resistant cells (158).

A large body of work has contributed to our understanding of the generation of DMs and HSRs (159–162). For example, Storlazzi et al. investigated the structures of *MYCN* amplifications by using eight neuroblastoma and two small cell carcinoma cell lines (162). The study provided evidence of generation of HSRs from DMs by an episome model wherein DNA segments were excised from a chromosome and then circularized and amplified to form DMs and chromosomally integrated to form HSRs. DMs are unstable and can be eliminated after drug treatment (163, 164); however, HSRs are more stable (165) (Fig. 1D and 3A). Amplified genes present on extrachromosomal DNA have been frequently observed in different tumor types (159, 166–168). The reversion of a malignant phenotype and cellular differentiation by the elimination of DMs has been shown extensively in a variety of tumors and cancer cell lines (167, 169, 170). Taken together, these observations demonstrate that transient gene amplifications can be an effective strategy for quick adaptation to selective pressures in tumor cells (Fig. 3A).

In a recent study by Nathanson et al., another example of drug-induced transient gene selection was demonstrated (Fig. 3B). In that study, oncogenes maintained on extrachromosomal DNA were transiently gained/lost in response to drug treatment (171). Glioblastoma patients harbor a constitutively active oncogenic variant of epidermal growth factor receptor (EGFR-vIII) that is formed by the in-frame deletion of exons 2 to 7 in the *EGFR* gene and found on extrachromosomal DNA (171, 172). The presence of EGFR-vIII makes tumor cells more sensitive to EGFR tyrosine kinase inhibitors (TKIs) (173). The continued treatment with EGFR TKIs (e.g., erlotinib) resulted in a loss of extrachromosomal EGFR-vIII, thus conferring resistance to the TKI. When the drug was withdrawn for a short period of time, there was an increase in EGFR-vIII on extrachromosomal DNA and, in turn, the cells were resensitized to erlotinib treatment (Fig. 3B). These data reiterate the reversibility of copy number gains and how transient copy number changes could impact chemotherapeutic response.

Furthermore, Nathanson and colleagues have suggested that instead of a continuous therapeutic regimen, a drug holiday during therapy might be a more effective mechanism to restore the sensitivity of tumor cells to drugs (171). These studies raise the possibility that chemotherapy could result in the selection of cells with gene amplifications, which allow them to survive under this drug-induced stress (Fig. 3). Therefore, understanding the mechanisms that result in transient or nonpermanent amplifications of *DHFR*, *EGFR*, and alike in cancer (Table 1) will have a profound impact on how we view copy number control as well as how we identify novel biomarkers and therapeutic targets for treating drug-resistant cancers.

TSSGs, TUMOR HETEROGENEITY, AND CANCER EVOLUTION

There are frequent gains/amplifications observed across cancer genomes, which are often thought to be permanent events (33, 160). However, a recent discovery from our laboratory (174, 175) suggested a possible mechanism for the intratumoral heterogeneity of copy number alterations observed in tumors. This recent discovery could also provide a molecular basis for the emergence of amplified drug resistance genes and enhanced cancer cell survival.

Chromatin modulation plays an important role in replication

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fidelity (176, 177). A recent study demonstrated that alterations in chromatin states could modulate copy number gains at distinct regions in the genome (175). KDM4A/JMJD2A demethylates trimethylated histone H3 lysines 9 and 36 (H3K9/36me3) to a dimethylated state (K3K9/36me2) (178–182). KDM4A overexpression promoted faster S-phase progression and altered replication timing at specific regions in the genome in a catalytically dependent manner (175, 183). The regulation of S phase and replication timing were conserved from *Caenorhabditis elegans* to human cells and were the result of dysregulating specific HP1 members in the genome (HPL-2 in *C. elegans* and HP1γ in human cells) (183).

Even though the S phase was faster in mammalian cells, the rate of cell proliferation was the same, which was consistent with the observed slowing into the G_2/M phase. This delayed G_2/M was not associated with major genome instability. However, KDM4A overexpression directly generated site-specific copy gains of regions affiliated with drug resistance (e.g., chr 1q21-22) by altering methylation states and heterochromatin association. KDM4A was enriched at these sites and promoted their rereplication. Furthermore, direct H3K9/36me3 interference promoted site-specific copy number gain events. This study demonstrated for the first time that an enzyme has the ability to directly regulate copy number gain at specific regions in the genome and that the chromatin/methylation states play an essential role in the process (175) (Fig. 3C).

Since the copy number gain regions are not permanent and are only generated and present during S phase, they have been termed transient site-specific copy gains (174, 175, 184). Currently, we do not know the exact sizes of the rereplicated fragments and whether there are cellular checkpoints/machinery involved in their clearance. In fact, different cells in a population have differentially amplified regions, and certain regions are mutually exclusive. Furthermore, the rate that these fragments are removed as cells move through S phase is different (174). It is important to determine the molecular features (e.g., presence of repetitive elements, insulators, and other regulatory machinery) at and surrounding the rereplicated and regions that gain copies. These molecular details will help establish whether unique sequence features or chromatin states have a predilection for rereplication and whether site-specific copy gains can be integrated in the genome.

Stabilization of KDM4A as a result of exposure to cellular triggers such as hypoxia also resulted in TSSGs in cell lines, tumors, and normal primary cells (Fig. 2, T cells) (174). In fact, these copy number gains were found to be conserved at a syntenic region in zebrafish cells subjected to hypoxia. The return of cells to normoxia resulted in the reversion of copy number gains to the baseline levels (Fig. 3C). Hence, generation of transient copy number gains could be an adaptive cellular response of cells to external stresses or stimuli. These data provide a mechanism for heterogeneity within a cell population even though the same genetic event occurred in the population.

The stabilization of KDM4A upon hypoxic exposure or inhibition or loss of microRNAs regulating KDM4A promoted copy number gains of the drug resistance oncogene *CKS1B* (111, 112, 121, 185), which had a concomitant increase in transcripts (174, 191). When cells were returned to normoxic conditions, both copy number and transcripts of *CKS1B* returned to normal levels. Finally, we demonstrated that succinate (a natural inhibitor for the KDM4 class of demethylases [186]), chemical inhibition or microRNA-targeted depletion of KDM4A blocked the copy number gains upon hypoxic exposure (174, 191). These data emphasize the impact that metabolites could have on copy number gain, but most importantly, they identify a mechanism for blocking their generation (Fig. 3C). Since drug resistance oncogenes were increased, the inhibition of KDM4A may provide a novel mechanism for modulating TSSGs and provide a method for reducing 1q21 drug resistance-associated cancers.

The fact that transient exposure to elevated KDM4A can promote copy number gain that is only present during S phase suggests that other mechanisms must be present to remove the TSSGs. Similar mechanisms may be involved in the removal of extrachomosomal *DHFR* and *EGFR* amplifications. The TSSG data support the notion that chromosomal regions with specific genes that confer a survival advantage are amplified to protect the cell. Selectively amplifying genes that confer distinct advantages related either to cell survival, metabolism of drugs, mounting responses to counteract drug sensitivity, or features promoting tumorigenesis could aid in the evolution/adaptation of cancer cells. The question remains as to whether the classical oncogenes (e.g., *EGFR*, *MYC*, *ERBB2*, etc.) (Table 1) are subjected to site-specific copy gains in tumors and subsequent retention upon genetic, intrinsic, or extrinsic exposure. Some extrinsic cues could be therapeutic or metabolic challenge, stress conditions (such as hypoxia, nutrient deprivation), and vasculature and extracellular matrix plasticity. Future studies investigating their impact on TSSGs and gene amplification will be critical.

Tumor heterogeneity. Tumor heterogeneity presents a major diagnostic and therapeutic challenge in the treatment of cancer. Indeed, recent sequencing efforts with next-generation sequencing helped in the tracing of clonal lineages in tumors (187, 188). Focal gains or losses of chromosomes can result in diversity among cells in a tumor population (intratumoral heterogeneity [189]) as well as between tumors (intertumoral heterogeneity [189]). For example, next-generation sequencing of five bladder tumors from patients with transitional cell carcinoma of the urinary bladder showed genomic rearrangements and mutational heterogeneity within tumors (188). Whole-exome sequencing of samples from 18 patients with chronic lymphocytic leukemia (CLL) revealed the emergence of subclones within selected populations of cells treated with chemotherapy (190). These populations of cells might be more fit than their pretreatment counterparts and could contribute to relapse after therapy. Thus, identifying the mutational landscape before and after chemotherapy could not only identify mechanisms of tumor relapse but also help to design effective therapeutic options for the elimination of dominant subclones arising after chemotherapeutic selection pressures.

Another mechanism contributing to intratumoral heterogeneity could be the regulation of TSSGs from KDM4A levels, oxygen concentrations, cell division rates, metabolites, and KDM4A inhibition. Cells could be cycling at different rates in a tumor population, thereby affecting the rate at which rereplicated fragments are generated (Fig. 3C). Differential levels of KDM4A expression, hypoxia levels, or metabolic status in cells within a tumor population could also generate copy number gains at different rates, thereby affecting heterogeneity. We hypothesize that the site-specific rereplication events could be one of the characteristics acquired in specific population of cells during subclonal divergence. Specific environmental, metabolic, or therapeutic stress condi-

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tions can produce site-specific chromosomal alterations in the subclonal populations, which could either be transient, persisting only when the signal is there, or could eventually become integrated elsewhere in the genome upon subsequent genetic/epigenetic changes. TSSGs within specific cell populations could either influence the emergence of the dominant subclone or could go hand in hand with the germ line mutations occurring during tumor evolution. Whether these events result in the emergence of the fittest clone that promotes survival and if these sets of "fit" cells clonally expand after a therapeutic challenge is a hypothesis that needs to be investigated.

CONCLUSIONS

CNVs influence the ability of normal cells to respond to physiological triggers and can serve as an adaptive strategy for a variety of responses, such as hypoxia, nutrient deprivation, toxic challenges, or cell survival and proliferation. Alterations in copy number often lead to diseases such as cancer, where the tumor cells can also coopt these aberrations as an adaptive response to amplify genes involved in chemotherapeutic resistance. It is important to determine whether the processes of generating copy number alterations under normal physiological, developmental, or pathological conditions are based on an active cell-directed and regulated mechanism or are the result of random aberrations that have occurred during cell division. Whether random or directed, it is important to understand that copy number changes are not always permanent. The recent discovery of a specific chromatin regulator controlling rereplication and site-specific copy number changes suggests that copy number changes can be regulated and are reversible. These transient site-specific copy gains may generate intratumoral heterogeneity that could have important consequences in chemotherapeutic sensitivity and patient outcome. Hence, identifying regulators of CNVs and delineating processes affected by CNVs will be important therapeutically.

ACKNOWLEDGMENTS

We thank members of the Whetstine laboratory for their critical comments on the manuscript. We also thank Mo Motamedi, Amity Manning, and Deepak Kumar Jha for helpful discussions.

Work related to this review is supported by funding to Johnathan R. Whetstine from the American Cancer Society (RSG-13-115-01-CCG) and the National Institutes of Health (CA059267 and R01GM097360). J.R.W. is a Tepper Family Massachusetts General Hospital Scholar and Leukemia and Lymphoma Scholar. J.R.W. is a recipient of an American Lung Cancer Discovery Award and an Innovation Award from the Alex Lemonade Stand Foundation. Sweta Mishra is supported by a Senior Research Training Fellowship from the American Lung Association.

FUNDING INFORMATION

HHS | National Institutes of Health (NIH) provided funding to Johnathan R. Whetstine under grant numbers GM097360 and CA059267. American Cancer Society (ACS) provided funding to Johnathan R. Whetstine under grant number RSG-13-115-01-CCG.

Additional support to Johnathan R. Whetstine and Sweeta Mishra was provided by the American Lung Association, the Alex Lemonade Stand Foundation, and the Leukemia and Lymphoma Society.

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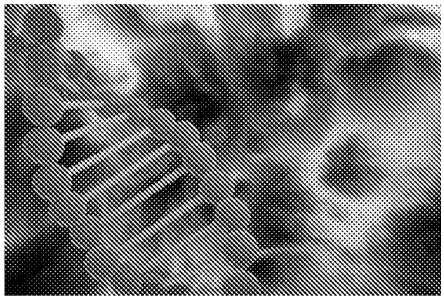
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Cancer Therapy Advisor

February 28, 2019

Genomic Profiling Using Guardant 360 Cell-Free DNA-Based Assay vs Tumor-Based Genotyping Assays in Advanced NSCLC

Susan Moench, PhD, PA-C



The Guardant360 assay is as effective as traditional tumor-based genotyping assays in identifying genomic alterations, according to researchers.

Tumor genomic profiling using the Guardant360 liquid biopsy-based test is as effective as standard-of-care tumor biopsy-based assays in identifying guideline-recommended biomarkers with matched U.S. Food and Drug Administration(FDA)-approved targeted therapies in patients with newly diagnosed advanced non-small cell lung cancer (NSCLC), according to a press announcement on results from the Noninvasive vs. Invasive Lung Evaluation (NILE) trial (ClinicalTrials.gov Identifier: NCT03615443).[1]

The Guardant360 assay is a commercially available next-generationsequencing test for identifying alterations in 73 genes from cell-free tumor DNA, and is performed on samples of blood plasma.[2] In contrast, tumor-based tests, such FoundationOne CDx, a next-generation sequencing assay, often provide the sequences of a larger number of genes (ie, 324 in FoundationOneCDx).[3] In addition, traditional tumor-based genotyping assays, which are not next-generation sequencing tests and typically interrogate a more limited number of genes, are frequently used in the setting of advanced NSCLC.[1] Nevertheless,tumor-based assays, unlike the Guardant360 assay, require a tumor biopsy and are invasive, which may be particularly important in the management of cancers such

as NSCLC which frequently involves multiple assessments of tumor DNA over time as a result of acquired resistance to targeted treatments.

The NILE trial is a multicenter,prospective study involving 282 patients with newly diagnosed, advanced NSCLC. The primary endpoint of the study is the proportion of tumor genomic alterations found in at least 1 of 7 predictive biomarkers (ie, *EGFR*, *ALK*, *ROS1*, *BRAF*,*MET*, *ERBB2*, and *RET*) recommended for testing in the National Comprehensive Cancer Network (NCCN) Guidelines for NSCLC using the Guardant360 assay compared with standard-of-care tumor-based genotyping assays.[1,4] The rationale for inclusion of the 7 predictive biomarkers in the NCCN NSCLC guidelines is that identification of alterations in these genes can guide treatment decisions related to use of targeted therapy for up to 30% of patients.[1,4]

Results of the NILE trial showed that mutations in at least 1 of the 7 guideline-recommended predictive biomarkers were identified in 77 and 60 patients tested using the Guardant360 assay and a traditional tissue-based genotyping assay, respectively(P <.0001). Of note, some of the tumor biopsy specimens were not suitable for testing. Although 12 of the 60 patients had an identified predictive biomarker in tumor alone (not seen with the Guardant360 assay), the number of patients with an identified predictive biomarker increased by 48% when the Guardant360 assay was performed in addition to tumor-based genotyping. Furthermore, in the specimens that were negative fora predictive biomarker by a tumor-based assay, addition of the Guardant360 assay increased the number of patients with an identified *KRAS* mutation, a prognostic biomarker, from 24 to 92.[1]

Importantly, a comparison of the alterations obtained with the 2 types of assays regarding mutations in 4 predictive biomarkers (*EGFR*, *ALK*, *ROS1*,and *BRAF*) for which there are FDA-approved targeted therapies, showed concordant results, with a positive predictive value of 100%. Of note, the median time to results was shorter using the Guardant 360 assay compared with the tumor-based assays (9 days vs 15 days,respectively; P <.0001).

The results of the NILE trial will be presented at the American Association for Cancer Research (AACR) Annual Meeting in Atlanta, Georgia, on April 2, 2019.

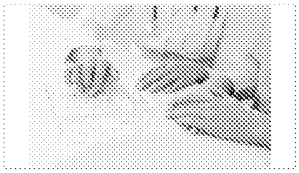
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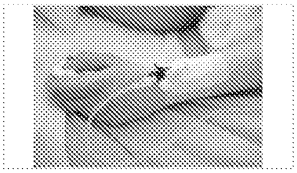
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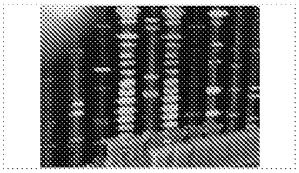
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An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage

Aaron M Newman[1,2,7], Scott V Bratman[1,3,7], Jacqueline To[3], Jacob F Wynne[3], Neville C W Eclov[3], Leslie A Modlin[3], Chih Long Liu[1,2], Joel W Neal[2], Heather A Wakelee[2], Robert E Merritt[4], Joseph B Shrager[4], Billy W Loo Jr[3], Ash A Alizadeh[1,2,5] & Maximilian Diehn[1,3,6]

Circulating tumor DNA (ctDNA) is a promising biomarker for noninvasive assessment of cancer burden, but existing ctDNA detection methods have insufficient sensitivity or patient coverage for broad clinical applicability. Here we introduce cancer personalized profiling by deep sequencing (CAPP-Seq), an economical and ultrasensitive method for quantifying ctDNA. We implemented CAPP-Seq for non–small-cell lung cancer (NSCLC) with a design covering multiple classes of somatic alterations that identified mutations in >95% of tumors. We detected ctDNA in 100% of patients with stage II–IV NSCLC and in 50% of patients with stage I, with 96% specificity for mutant allele fractions down to ~0.02%. Levels of ctDNA were highly correlated with tumor volume and distinguished between residual disease and treatment-related imaging changes, and measurement of ctDNA levels allowed for earlier response assessment than radiographic approaches. Finally, we evaluated biopsy-free tumor screening and genotyping with CAPP-Seq. We envision that CAPP-Seq could be routinely applied clinically to detect and monitor diverse malignancies, thus facilitating personalized cancer therapy.

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Analysis of ctDNA has the potential to revolutionize detection and monitoring of tumors. Noninvasive access to cancer-derived DNA is particularly attractive for solid tumors, which cannot be repeatedly sampled without invasive procedures. In NSCLC, PCR-based assays have been used to detect recurrent point mutations in genes such as *KRAS* (encoding kirsten rat sarcoma viral oncogene homolog) or *EGFR* (encoding epidermal growth factor receptor) in plasma DNA[1–4], but the majority of patients lack mutations in these genes. Recently, approaches employing massively parallel sequencing have been used to detect ctDNA[5–12]. However, the methods reported to date have been limited by modest sensitivity[13], applicability to only a minority of patients, the need for patient-specific optimization and/or cost. To overcome these limitations, we developed a new strategy for analysis of ctDNA. Our approach, called CAPP-Seq, combines optimized library preparation methods for low DNA input masses with a multiphase bioinformatics approach to design a 'selector' consisting of biotinylated DNA oligonucleotides that target recurrently mutated regions in the cancer of interest. To monitor ctDNA, the selector is applied to tumor DNA to identify a patient's cancer-specific genetic aberrations and then directly to circulating DNA to quantify them (**Fig. 1a**). Here we demonstrate the technical performance and explore the clinical utility of CAPP-Seq in patients with early- and advanced-stage NSCLC.

RESULTS

Design of a CAPP-Seq selector for NSCLC

For the initial implementation of CAPP-Seq, we focused on NSCLC, although our approach is generalizable to any cancer for which recurrent mutations have been identified. To design a selector for NSCLC (**Fig. 1b**, **Supplementary Table 1** and Online Methods), we began by including exons covering recurrent mutations in potential driver genes from the Catalogue of Somatic Mutations in Cancer (COSMIC)[14] and other sources[15,16]. Next, using whole-exome sequencing (WES) data from 407 patients with NSCLC profiled by The Cancer Genome Atlas (TCGA), we applied an iterative algorithm to maximize the number of missense mutations per patient while minimizing selector size (**Supplementary Fig. 1** and **Supplementary Table 1**).

Approximately 8% of NSCLCs harbor rearrangements involving the receptor tyrosine kinase genes *ALK* (encoding anaplastic lymphoma receptor tyrosine kinase), *ROS1* (encoding c-ros oncogene 1 tyrosine kinase) or *RET* proto-oncogene[17–21]. To utilize the low false detection rate inherent in the unique junctional sequences of structural rearrangements[5,6], we included the introns and exons spanning recurrent fusion breakpoints in these genes in the final design phase (**Fig. 1b**). To detect fusions in tumor and plasma DNA, we developed a breakpoint-mapping algorithm optimized for ultradeep coverage data (**Supplementary Methods**). Application of this algorithm to next-generation sequencing (NGS) data from two NSCLC cell lines known to harbor fusions with previously uncharacterized

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Received 15 August 2013; accepted 6 November 2013; published online 6 April 2014; doi:10.1038/nm.3519

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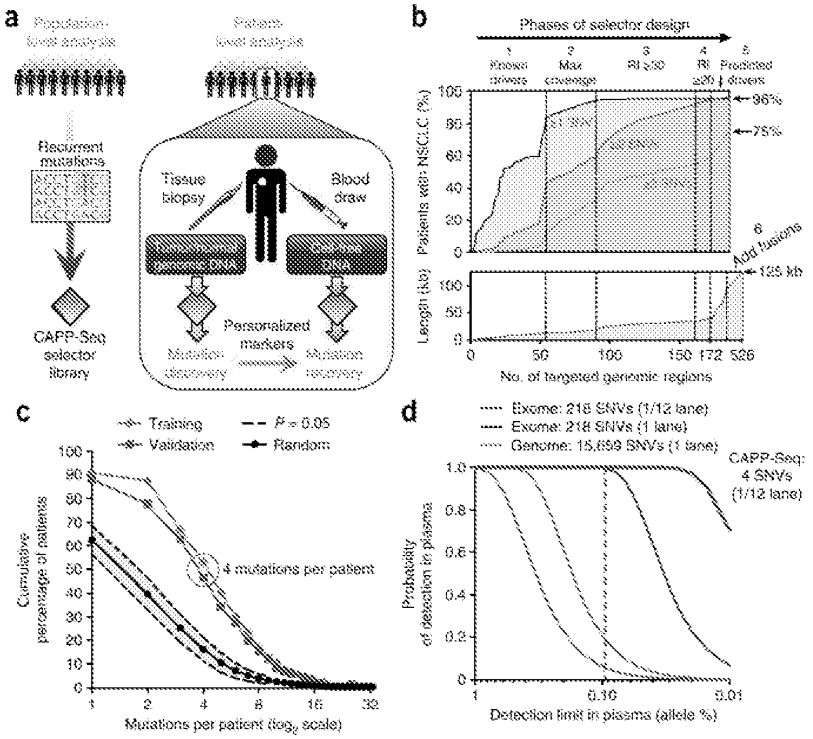


Figure 1 Development of CAPP-Seq. (**a**) Schematic depicting design of CAPP-Seq selectors and their application for assessing ctDNA. (**b**) Multiphase design of the NSCLC selector. Phase 1: genomic regions harboring known and suspected driver mutations in NSCLC are captured. Phases 2–4: addition of exons containing recurrent SNVs using WES data from lung adenocarcinomas and squamous cell carcinomas from TCGA ($n = 407$). Recurrence index (RI) is equal to total unique patients with mutations covered per kb of exon. Phases 5 and 6: addition of exons of predicted NSCLC drivers[15,16] and introns and exons harboring breakpoints in rearrangements involving *ALK*, *ROS1* and *RET*. Bottom: increase of selector length during each design phase. (**c**) Analysis of the number of SNVs per lung adenocarcinoma covered by the NSCLC selector in the TCGA WES cohort (Training; $n = 229$) and an independent lung adenocarcinoma WES data set (Validation; $n = 183$)[20]. Results are compared to selectors randomly sampled from the exome ($P < 1.0 \times 10^{-6}$ for the difference between random selectors and the NSCLC selector; *Z*-test, Online Methods). (**d**) Analytical modeling of CAPP-Seq, whole-exome sequencing and whole-genome sequencing for different detection limits of ctDNA in plasma. Calculations are based on the median number of mutations detected per NSCLC for CAPP-Seq (i.e., 4) and the reported number of mutations in NSCLC exomes and genomes[21]. Additional details, including assumed sequencing throughput (i.e., bases) per lane, are described in Online Methods. The vertical dashed line represents the median fraction of ctDNA detected in plasma from patients in this study.

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breakpoints[22,23] readily identified the breakpoints at nucleotide resolution (**Supplementary Fig. 2**).

Collectively, the NSCLC selector design targets 521 exons and 13 introns from 139 recurrently mutated genes, in total covering ~125 kb (**Fig. 1b**). Within this small target (0.004% of the human genome), the selector identifies a median of four single nucleotide variants (SNVs) and covers 96% of patients with lung adenocarcinoma or squamous cell carcinoma. To validate the number of mutations covered per tumor, we examined the selector region in WES data from an independent cohort of 183 patients with lung adenocarcinoma[20]. The selector covered 86% of patients with a median of four SNVs per patient, approximately fourfold more than would be expected from random sampling of the exome ($P < 1.0 \times 10^{-6}$; **Fig. 1c**), thus validating our selector design algorithm.

Methodological optimization and performance assessment

We performed deep sequencing with the NSCLC selector to achieve ~10,000× coverage (preduplication removal) based on considerations of sequencing depth, median number of reporters and ctDNA detection limit (**Fig. 1d**). We profiled a total of 90 samples, including two NSCLC cell lines, 17 primary tumor samples with matched peripheral blood leukocytes (PBLs) and 40 plasma samples from 18 human subjects, including 5 healthy adults and 13 patients with NSCLC (**Supplementary Table 2**). To assess and optimize selector performance, we first applied it to circulating DNA purified from healthy control plasma and observed efficient and uniform capture of genomic DNA (**Supplementary Table 2**). Sequenced plasma DNA fragments had a median length of ~170 bp (**Fig. 2a**), which closely corresponds to the length of DNA contained within a chromatosome[24]. By optimizing library preparation from small quantities of plasma DNA, we increased recovery efficiency by >300% and decreased bias for libraries constructed from as little as 4 ng of DNA (**Supplementary Fig. 3**). Consequently, fluctuations in sequencing depth were minimal (**Fig. 2b,c**).

The detection limit and accuracy of CAPP-Seq are affected by (*i*) the input number and recovery rate of circulating DNA molecules, (*ii*) sample cross-contamination, (*iii*) potential allelic bias in the capture reagent and (*iv*) PCR or sequencing errors. We examined each of these elements in turn. First, by comparing the number of input DNA molecules per sample with estimates of library complexity (**Supplementary Fig. 4a** and **Supplementary Methods**), we calculated a circulating DNA molecule recovery rate of ≥49% (**Supplementary Table 2**). This was in agreement with molecule recovery yields calculated following PCR (**Supplementary Fig. 4b**). Second, by analyzing patient-specific homozygous single nucleotide polymorphisms (SNPs) across samples, we found cross-contamination of ~0.06% in multiplexed plasma DNA (**Supplementary Fig. 4c** and **Supplementary Methods**), prompting us to exclude any tumor-derived SNV from further analysis if found as a germline SNP in another profiled patient. Next, we evaluated the allelic skew in heterozygous germline SNPs within patient PBL samples and observed minimal bias toward capture of reference alleles (**Supplementary Fig. 4d**). Finally, we analyzed the distribution of nonreference alleles across the selector for the 40 plasma DNA samples, excluding tumor-derived SNVs and germline SNPs. We found mean and median background rates of 0.006% and 0.0003%, respectively (**Fig. 2d**), both considerably lower than previously reported NGS-based methods for ctDNA analysis[6,10].

Nongermline plasma DNA could be present in the absence of cancer owing to contributions from preneoplastic cells from diverse

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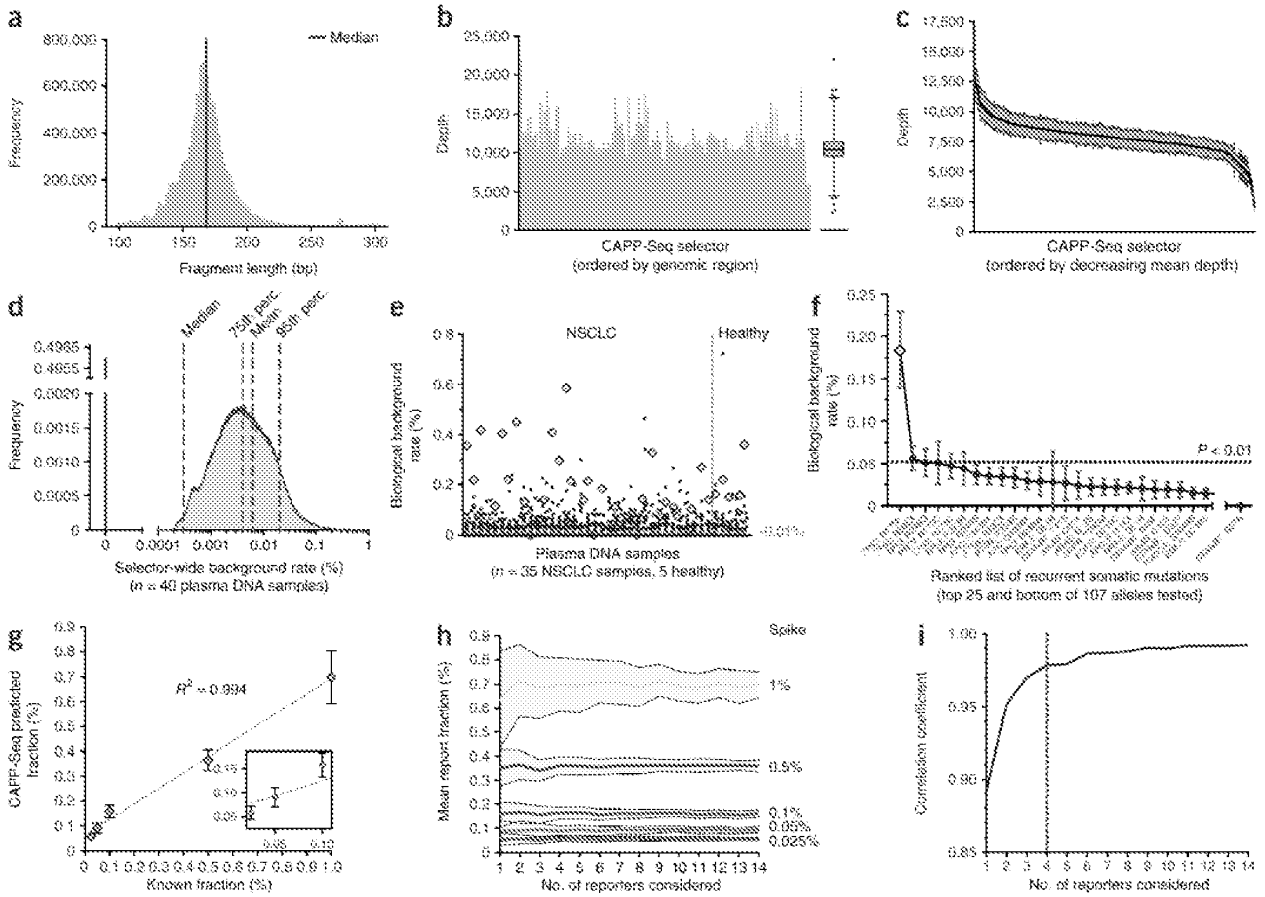


Figure 2 Analytical performance. (**a**–**c**) Quality parameters from a representative CAPP-Seq analysis of plasma DNA, including length distribution of sequenced circulating DNA fragments (fragment counts are represented on the *y* axis) (**a**) and depth of sequencing coverage (*y* axis) across all genomic regions in the selector (**b**) (details of all plasma DNA samples sequenced are shown in **Supplementary Table 2**). (**c**) Variation in sequencing depth (*y* axis) across plasma DNA samples from four patients. Orange envelope represents mean ± s.e.m. (**d**) Analysis of allelic background rate for 40 plasma DNA samples collected from 13 patients with NSCLC and 5 healthy individuals. The *y* axis denotes the fraction of all alleles and selector positions tested. Details are given in **Supplementary Methods**. Perc., percentile. (**e**) Analysis of biological background in **d** focusing on 107 recurrent somatic mutations from a previously reported SNaPshot panel[25]. Mutations found in a given patient's tumor were excluded. The mean frequency over all subjects was ~0.01%. A single outlier mutation (*TP53* R175H) is indicated by an yellow diamond. (**f**) Individual mutations from **e** ranked by most to least recurrent, according to mean frequency across the 40 plasma DNA samples. The *P* value threshold of 0.01 (dotted line) corresponds to the 99th percentile of global selector background in **d**. (**g**) Dilution series analysis of expected versus observed frequencies of mutant alleles using CAPP-Seq ($n = 14$ reporter alleles). Five concentrations of fragmented HCC78 DNA spiked into control circulating DNA are shown. (**h**) Analysis of the effect of the number of SNVs considered on the estimates of fractional abundance using data from **g**. Data are presented as means ± 95% confidence interval. (**i**) Analysis of the effect of the number of SNVs considered on the mean correlation coefficient between expected and observed cancer fractions (blue solid line) using data from panel **g**. 95% confidence intervals are shown for **e**,**f**. Statistical variation for **g** is shown as mean ± s.e.m.

tissues, and such 'biological' background may also affect CAPP-Seq sensitivity. We hypothesized that biological background, if present, would be particularly high for recurrently mutated positions in known cancer driver genes and therefore analyzed mutation rates of 107 cancer-associated SNVs[25] in all 40 plasma samples, excluding somatic mutations found in each patient's tumor. Although the median fractional abundance was comparable to the global selector background (~0%), the mean was marginally higher at ~0.01% (**Fig. 2e**). Notably, we detected one mutational hotspot (tumor suppressor *TP53*, R175H) at a median frequency of ~0.18% across all plasma DNA samples, including those from patients and healthy subjects (**Fig. 2f**). As we observed the frequency of this *TP53* mutant allele to be significantly above global background ($P < 0.01$), we hypothesize that it reflects true biological clonal heterogeneity and thus excluded it as a potential reporter. To address background more generally, we also normalized for allele-specific differences in background rate when assessing the significance of ctDNA detection (**Supplementary Methods**). As a result, we found that biological background is not a major factor affecting ctDNA quantitation at detection limits above ~0.01%.

Next, we empirically benchmarked the detection limit and linearity of CAPP-Seq (**Fig. 2g** and **Supplementary Fig. 5a**). We accurately detected defined inputs of NSCLC DNA at fractional abundances between 0.025% and 10% with high linearity ($R^2 \geq 0.994$). We observed only marginal improvements in error metrics above a threshold of four SNP reporters (**Fig. 2h**,**i** and **Supplementary Fig. 5b**,**c**), which is equivalent to the median number of SNVs per tumor identified by the selector. Moreover, the fractional abundance of fusion breakpoints,

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Table 1 Patient characteristics and pretreatment CAPP-Seq monitoring results

									Fusion		Pretreatment		
Case	Age	Sex	Histology	Stage	TNM	Smoking history	No. of SNVs (nonsilent)	Indels	*ALK* or *ROS1*	Partner	ctDNA (%)	ctDNA (pg ml^{-1})	Tumor (ml)
P12	86	F	SCC	IA	T1bN0M0	Heavy	6 (3)	1			ND	ND	5.5
P1	66	M	Adeno	IB	T2aN0M0	Heavy	12 (3)	4			0.025	1.9	23.1
P16	82	F	Adeno	IB	T2aN0M0	Heavy	26 (5)	2			0.019	2.5	22.5
P17	85	F	Adeno	IB	T2aN0M0	Heavy	2 (2)	0			ND	ND	10.2
P13	90	F	SCC	IIB	T3N0M0	Heavy	5 (4)	0			1.78	269.8	339.3
P2	61	M	Large cell	IIIA	T3N1M0	Heavy	12 (3)	1			0.896	64.7	23.1
P3	67	F	Adeno	IIIB	T1bN3M0	Light	1 (1)	0			0.095	16.2	7.9
P14	55	M	Adeno	IIIB	T1aN3M0	Heavy	8 (5)	0			0.05	10.2	5.2
P15	41	M	Adeno	IIIB	T3N3M0	Light	25 (10)	1			0.58	108.1	121.8
P4	47	F	Adeno	IV	T2aN2M1b	Heavy	3 (2)	0			0.039	2.1	12.4
P5	49	F	Adeno	IV	T1bN0M1a	None	4 (3)	0			3.2	143.8	82.1
P6	54	M	Adeno	IV	T3N2M1b	None	3 (2)	0	*ALK*	*KIF5B*	1.0	350.2	NA
P9	49	M	Adeno	IV	T4N3M1a	None	0	0	*ALK*	*EML4*	0.04	3.8	66.2
									ROS1	*MKK, FYN*			
P10	35	F	Adeno	IIIA	T4N0M0	None	0	0	*ROS1*	*SLC34A2*	--	--	--
P11	38	F	Adeno	IIIA	T3N2M0	None	2 (1)	0	*ROS1*	*CD74*	--	--	--
P7	50	M	Adeno	IV	T1aN2M1b	Light	0	0	*ALK*	*EML4*	--	--	--
P8	48	F	Adeno	IV	T4N0M1b	None	1 (0)	0	*ALK*	*EML4*	--	--	--

ND, mutant DNA was not detected above background (Online Methods); NA, tumor volume could not be reliably assessed. Dashes indicate a plasma sample was not available. Smoking history, ≥20 pack years (Heavy), >0 and <20 pack years (Light). SCC, small cell cancer; Adeno, adenocarcinoma; TNM, tumor, node and metastasis classification system. Additional details are provided in **Supplementary Tables 3** and **4**.

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insertions and deletions (indels) and copy number alterations (CNAs) correlated highly with expected concentrations ($R^2 \geq 0.97$; **Supplementary Fig. 5d**).

Somatic mutation detection and tumor burden quantitation

We next applied CAPP-Seq to the discovery of somatic mutations in tumor samples collected from 17 patients with NSCLC (**Table 1** and **Supplementary Table 3**), including formalin-fixed surgical resections, needle biopsy specimens and malignant pleural fluid. At a mean sequencing depth of ~5,000× (preduplicate removal) in tumor and paired germline samples (**Supplementary Table 2**), we detected 100% of previously identified SNVs and fusions and discovered many additional somatic variants (**Table 1** and **Supplementary Table 3**). Moreover, we characterized breakpoints at base-pair resolution and identified partner genes for each of eight known fusions involving *ALK* or *ROS1* (**Supplementary Fig. 2**). Tumors containing fusions were almost exclusively from never-smokers and contained fewer SNVs than those lacking fusions, as expected[21] (**Supplementary Fig. 2**). Excluding patients with fusions, we identified a median of six SNVs (three missense) per patient (**Table 1**), in line with our selector design-stage predictions (**Fig. 1b,c**).

Next, we assessed the sensitivity and specificity of CAPP-Seq for disease monitoring and minimal residual disease detection using plasma samples from 5 healthy controls and 35 samples collected from 13 patients with NSCLC (**Table 1** and **Supplementary Table 4**). We integrated information content across multiple instances and classes of somatic mutations into a ctDNA detection index. This index is analogous to a false-positive rate and is based on a decision tree in which fusion breakpoints take precedence because of their nonexistent background and in which *P* values from multiple reporter types are integrated (Online Methods). When we applied this approach in a receiver operating characteristic (ROC) analysis, CAPP-Seq achieved an area under the curve (AUC) of 0.95, with maximal sensitivity and specificity of 85% and 96%, respectively, for all plasma DNA samples from untreated patients and healthy controls. Sensitivity among patients with stage I tumors was 50%, and among those with stage II–IV tumors, it was 100%, with a specificity for both groups of 96% (**Fig. 3a,b**). Moreover, when considering both pre- and post-treatment samples, CAPP-Seq exhibited robust performance, with AUC values of 0.89 for all stages and 0.91 for stages II–IV ($P < 0.0001$, *Z*-test, Online Methods; **Supplementary Fig. 6**). Furthermore, by adjusting the ctDNA detection index, we could increase specificity up to 98%

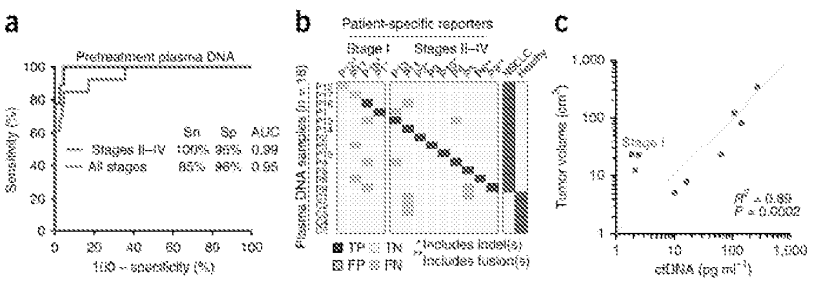


Figure 3 Sensitivity and specificity analysis. (**a**) ROC analysis of plasma DNA samples from pretreatment samples and healthy controls, divided into all stages ($n = 13$ patients) and stages II–IV ($n = 9$ patients). AUC values are significant at $P < 0.0001$ (*Z*-test, Online Methods). Sn, sensitivity; Sp, specificity. (**b**) Raw data related to **a**. TP, true positive; FP, false positive; TN, true negative; FN, false negative. (**c**) Concordance between tumor volume, measured by CT or PET-CT, and concentration (pg ml^{-1}) of ctDNA from pretreatment samples ($n = 9$), measured by CAPP-Seq. Patients P6 and P9 were excluded owing to inability to accurately assess tumor volume and differences related to the capture of fusions, respectively (**Supplementary Methods**). Of note, linear regression was performed in non-log space; the log-log axes and dashed diagonal line are for display purposes only.

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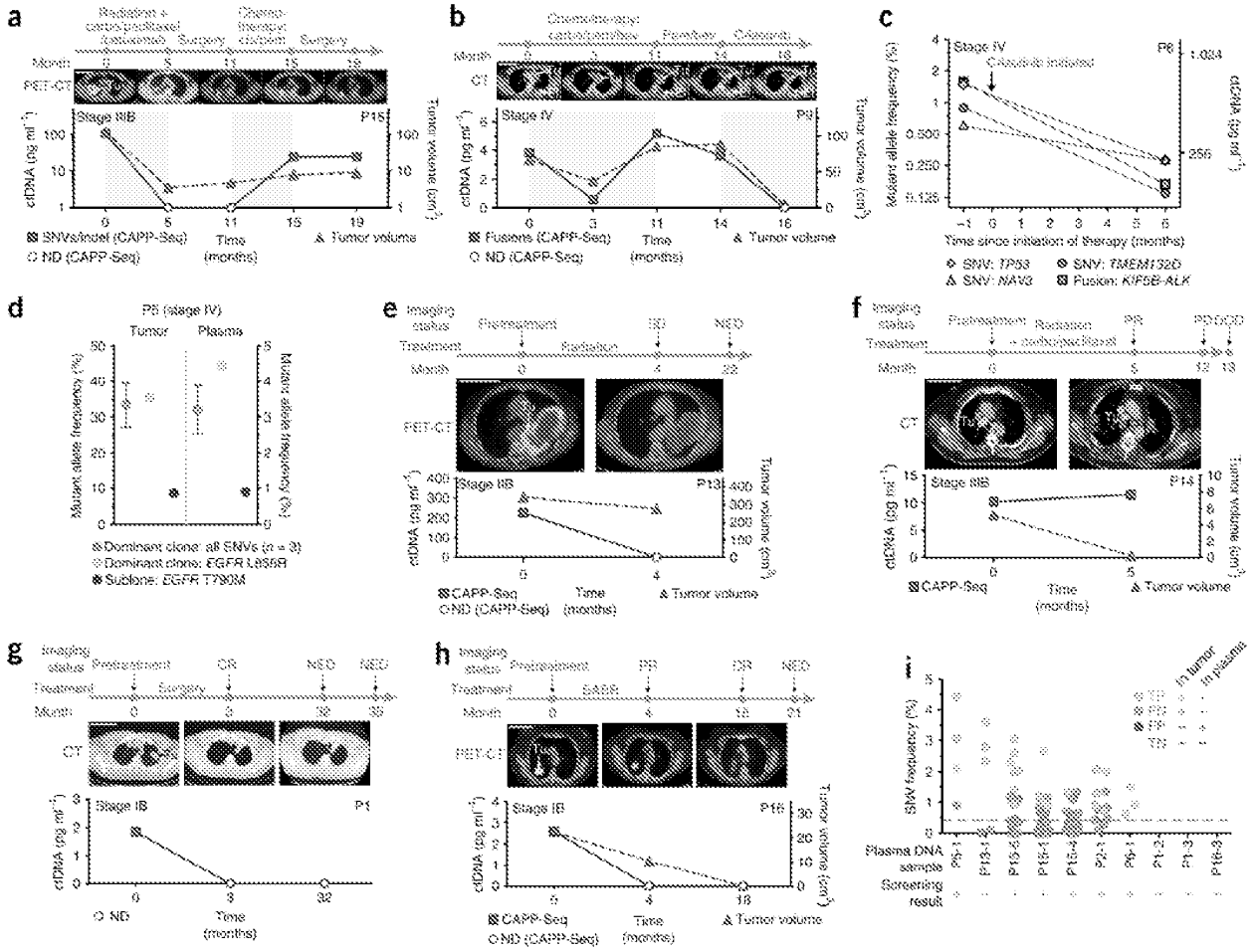


Figure 4 Noninvasive detection and monitoring of ctDNA. (**a**–**h**) Disease monitoring using CAPP-Seq. Carboplatin (carbo), paclitaxel, cetuximab, cisplatin (cis), pemetrexed (pem), bevacizumab (bev), or crizotinib were administered as combination therapies as indicated. (**a**,**b**) Disease burden changes in response to treatment in a patient with stage IIIB NSCLC using SNVs and an indel (SNVs/indel) (**a**) and a patient with stage IV NSCLC using three rearrangement breakpoints (**b**). Tu, tumor; Ef, pleural effusion; ND, not detected. (**c**) Concordance between different reporters (SNVs and a fusion) in a patient with stage IV NSCLC. *TMEM132D*, transmembrane protein 132D gene. (**d**) Detection of a subclonal *EGFR* T790M resistance mutation in a patient with stage IV NSCLC. The fractional abundance of the dominant clone and T790M-containing clone are shown in the primary tumor (left) and plasma samples (right). (**e**,**f**) CAPP-Seq results from post-treatment plasma DNA samples are predictive of clinical outcomes in a patient with stage IIB NSCLC (**e**) and a patient with stage IIIB NSCLC (**f**). SD, stable disease; PD, progressive disease; PR, partial response; NED, no evidence of disease; DOD, dead of disease. (**g**,**h**) Monitoring of tumor burden following complete tumor resection (**g**) and SABR (**h**) for two patients with stage IB NSCLC. CR, complete response. (**i**) Exploratory analysis of the potential application of CAPP-Seq for biopsy-free tumor genotyping or cancer screening. All plasma DNA samples from patients in **Table 1** were examined for the presence of mutant allele outliers without knowledge of the primary tumor mutations (**Supplementary Methods**); samples with detectable mutations are shown, along with three samples assumed to be cancer-negative (P1-2, P1-3 and P16-3; **Supplementary Methods**). The number following the hyphen in each sample (e.g., -1) represents the plasma time point (**Supplementary Table 4**). The lowest fraction of ctDNA among positive samples was ~0.4% (dashed horizontal line). Data in **d** are expressed as mean ± s.e.m. Scale bars (**a**,**b**,**e**–**h**), 10 cm.

while still capturing two-thirds of all cancer-positive samples and three-fourths of stage II–IV cancer-positive samples (**Supplementary Fig. 6**). Thus, CAPP-Seq can achieve robust assessment of tumor burden and can be tuned to deliver a desired sensitivity and specificity.

Monitoring of NSCLC tumor burden in plasma samples

We next asked whether significantly detectable levels of ctDNA correlate with radiographically measured tumor volumes and clinical responses to therapy. Fractions of ctDNA detected in plasma by SNV and/or indel reporters ranged from ~0.02% to 3.2% (**Table 1**), with a median of ~0.1% in pretreatment samples. Absolute levels of ctDNA in pretreatment plasma significantly correlated with tumor volume as measured by computed tomography (CT) and positron emission tomography (PET) imaging ($R^2 = 0.89$, $P = 0.0002$; **Fig. 3c**).

To determine whether ctDNA concentrations reflect disease burden in longitudinal samples, we analyzed plasma DNA from three patients with advanced NSCLC undergoing distinct therapies (**Fig. 4a**–**c**). As in pretreatment samples, ctDNA levels highly correlated with tumor volumes during therapy ($R^2 = 0.95$ for patient 15 (P15); $R^2 = 0.85$ for P9). This behavior was observed whether the mutation types measured were a collection of SNVs and an indel (P15, **Fig. 4a**), multiple fusions (P9, **Fig. 4b**) or SNVs and a fusion (P6, **Fig. 4c**). Of note, in

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one patient (P9), we identified both a classic *EML4-ALK* fusion and two previously unreported fusions involving *ROS1*: *FYN-ROS1* and *ROS1-MKX* (**Supplementary Fig. 2**). All fusions were confirmed by quantitative PCR (qPCR) amplification of genomic DNA and were independently recovered in plasma samples (**Supplementary Table 4**). To the best of our knowledge, this is the first observation of *ROS1* and *ALK* fusions in the same individual with NSCLC.

We designed the NSCLC CAPP-Seq selector to detect multiple SNVs per tumor. In one patient (P5), this design allowed us to identify a dominant clone with an activating *EGFR* mutation as well as an erlotinib-resistant subclone with a 'gatekeeper' *EGFR* T790M mutation[26]. The ratios between clones were identical in a tumor biopsy and in simultaneously sampled plasma (**Fig. 4d**), demonstrating that our method has potential for detecting and quantifying clinically relevant subclones.

Patients with stage II or III NSCLC undergoing definitive radiotherapy often have surveillance CT or PET-CT scans that are difficult to interpret owing to radiation-induced inflammatory and fibrotic changes in the lung and surrounding tissues. For patient P13, who was treated with radiotherapy for stage IIB NSCLC, follow-up imaging showed a large mass that was interpreted to represent residual disease. However, ctDNA at the same time point was undetectable (**Fig. 4e**), and the patient remained disease free 22 months later, which supports the ctDNA result. Another patient (P14) was treated with chemoradiotherapy for stage IIIB NSCLC, and follow-up imaging revealed a near-complete response (**Fig. 4f**). However, the ctDNA concentration slightly increased following therapy, suggesting progression of occult microscopic disease. Indeed, clinical progression was detected 7 months later, and the patient ultimately succumbed to NSCLC. These data highlight the promise of ctDNA analysis for identifying patients with residual disease after therapy.

We next asked whether the low detection limit of CAPP-Seq would allow monitoring in early-stage NSCLC. Patients P1 (**Fig. 4g**) and P16 (**Fig. 4h**) underwent surgery and stereotactic ablative radiotherapy (SABR)[27], respectively, for stage IB NSCLC. We detected ctDNA in pretreatment plasma of patient P1 but not at 3 or 32 months following surgery, which suggests that this patient was free of disease and probably cured[28]. For patient P16, the initial surveillance PET-CT scan following SABR showed a residual mass that was interpreted to represent either residual tumor or postradiotherapy inflammation. We detected no evidence of residual disease by ctDNA, supporting the latter hypothesis, and the patient remained free of disease at last follow-up 21 months after therapy. Taken together, these results demonstrate the potential utility of CAPP-Seq for measuring tumor burden in early- and advanced-stage NSCLC and for monitoring ctDNA during distinct types of therapy.

Biopsy-free cancer screening and tumor genotyping

Finally, we explored whether CAPP-Seq analysis of ctDNA could potentially be used for cancer screening and biopsy-free tumor genotyping. As proof of principle, we blinded ourselves to the mutations present in each patient's tumor and applied a new statistical method to test for the presence of cancer DNA in each plasma sample in our cohort (**Supplementary Fig. 7**). By implementing our cancer screening method for high specificity, we correctly classified 100% of patient plasma samples with ctDNA above fractional abundances of 0.4% with a false-positive rate of 0% (**Fig. 4i** and **Supplementary Methods**). CAPP-Seq could therefore potentially improve upon the low positive predictive value of low-dose CT screening in patients at high risk of developing NSCLC[29].

Separately, when we specifically examined the ability of CAPP-Seq to noninvasively detect actionable mutations in *EGFR* and *KRAS*[25], we correctly identified 100% of mutations at allelic fractions greater than 0.1% with 99% specificity. CAPP-Seq may therefore have utility for biopsy-free tumor genotyping in patients with locally advanced or metastatic NSCLC. However, methodological improvements will be required to detect and genotype stage I tumors without prior knowledge of tumor genotype.

DISCUSSION

In this study, we present CAPP-Seq as a new method for ctDNA quantitation. Its key features include high sensitivity and specificity, lack of a need for patient-specific optimization and coverage of nearly all patients with NSCLC. To our knowledge, CAPP-Seq is the first NGS-based method for ctDNA analysis that achieves both an ultralow detection limit and broad patient coverage at a reasonable cost. Our approach also reduces the potential impact of stochastic noise and biological variability (for example, mutations near the detection limit or subclonal tumor evolution) on tumor burden quantitation by integrating information content across multiple instances and classes of somatic mutations. These features facilitated the detection of minimal residual disease and ctDNA quantitation from stage I NSCLC tumors. Although we focused on NSCLC, our method could be applied to any malignancy for which recurrent mutation data are available.

In many patients, levels of ctDNA are considerably lower than the detection thresholds of previously described sequencing-based methods[13]. For example, pretreatment ctDNA concentration is <0.5% in the majority of patients with lung and colorectal carcinomas[1,30,31]. Following therapy, ctDNA concentrations typically drop, thus requiring even lower detection thresholds. Previously published ctDNA detection methods employing amplicon[8,10,11], whole-exome[12] or whole-genome[9,24,32,33] sequencing would not be sensitive enough to detect ctDNA in most patients with NSCLC, even at tenfold or greater sequencing costs (**Fig. 1d** and **Supplementary Fig. 8**).

To further expand the potential clinical applications of ctDNA quantitation, additional gains in the detection threshold are desirable. Potential approaches include using barcoding strategies that suppress PCR errors resulting from library preparation[34,35] and increasing the amount of plasma used for ctDNA analysis above the average of ~1.5 ml used in our study. A second limitation of CAPP-Seq is the potential for inefficient capture of fusions, which could lead to underestimates of tumor burden (for example, P9; **Supplementary Methods**). However, this bias can be analytically addressed when other reporter types are present (for example, P6; **Supplementary Table 4**). Finally, although we found that CAPP-Seq could quantitate CNAs, our current selector design did not prioritize these types of aberrations. We anticipate that adding coverage for certain CNAs will prove useful for monitoring various types of cancers.

In summary, targeted hybrid capture and high-throughput sequencing of plasma DNA allows for highly sensitive and noninvasive detection of ctDNA in the vast majority of patients with NSCLC at low cost. CAPP-Seq could therefore be routinely applied clinically and has the potential for accelerating the personalized detection, therapy and monitoring of cancer. We anticipate that CAPP-Seq will prove valuable in a variety of clinical settings, including the assessment of cancer DNA in alternative biological fluids and specimens with low cancer cell content.

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METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Raw sequencing data were deposited in the Sequence Read Archive with accession number SRP040228.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank S. Quake and members of his lab for suggestions and N. Neff for technical assistance. This work was supported by the US Department of Defense (M.D., A.A.A., A.M.N.), the US National Institutes of Health Director's New Innovator Award Program (M.D.; 1-DP2-CA186569), the Ludwig Institute for Cancer Research (M.D., A.A.A.), the Radiological Society of North America (S.V.B.; #RR1221), an Association of American Cancer Institutes Translational Cancer Research Fellowship (S.V.B.) and a grant from both the Siebel Stem Cell Institute and the Thomas and Stacey Siebel Foundation (A.M.N.). A.A.A. and M.D. are supported by Doris Duke Clinical Scientist Development Awards.

AUTHOR CONTRIBUTIONS

A.M.N., S.V.B., A.A.A. and M.D. developed the concept, designed the experiments, analyzed the data and wrote the manuscript. S.V.B. performed the molecular biology experiments, and A.M.N. performed the bioinformatics analyses. C.L.L. helped develop analytical pipeline software. S.V.B., J.T., J.F.W., N.C.W.E., L.A.M., J.W.N., H.A.W., R.E.M., J.B.S., B.W.L. Jr. and M.D. provided patient specimens. A.A.A. and M.D. contributed equally as senior authors. All authors commented on the manuscript at all stages.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Patient selection. Between April 2010 and June 2012, patients undergoing treatment for newly diagnosed or recurrent NSCLC enrolled in a study approved by the Stanford University Institutional Review Board and provided informed consent. Enrolled patients had not received blood transfusions within 3 months of blood collection. Patient characteristics are listed in **Supplementary Table 3**. All treatments and radiographic examinations were performed as part of standard clinical care. Volumetric measurements of tumor burden were based on visible tumor on CT and calculated according to the ellipsoid formula: (length/2) × width^2.

Sample collection and processing. Peripheral blood from patients was collected in EDTA Vacutainer tubes (Becton Dickinson). Blood samples were processed within 3 h of collection. Plasma was separated by centrifugation at 2,500*g* for 10 min, transferred to microcentrifuge tubes and centrifuged at 16,000*g* for 10 min to remove cell debris. The cell pellet from the initial spin was used for isolation of germline genomic DNA from PBLs with the DNeasy Blood & Tissue Kit (Qiagen). Matched tumor DNA was isolated from formalin-fixed, paraffin-embedded specimens or from the cell pellet of pleural effusions. Genomic DNA was quantified by Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen).

Cell-free DNA purification and quantification. Circulating DNA was isolated from 1–5 mL plasma with the QIAamp Circulating Nucleic Acid Kit (Qiagen). The concentration of purified plasma DNA was determined by qPCR using an 81-bp amplicon on chromosome 1 (ref. 24) and a dilution series of intact male human genomic DNA (Promega) as a standard curve. Power SYBR Green was used for qPCR on a HT7900 Real Time PCR machine (Applied Biosystems), using standard PCR thermal cycling parameters.

Next-generation sequencing library construction. Indexed Illumina NGS libraries were prepared from plasma DNA and shorn tumor, germline and cell line genomic DNA. For patient plasma DNA, 7–32 ng DNA were used for library construction without additional fragmentation. For tumor, germline and cell line genomic DNA, 69–1,000 ng DNA was sheared before library construction with a Covaris S2 instrument using the recommended settings for 200-bp fragments. Details are provided in **Supplementary Table 2**.

The NGS libraries were constructed using the KAPA Library Preparation Kit (Kapa Biosystems) employing a DNA polymerase possessing strong 3′→5′ exonuclease (or proofreading) activity and displaying the lowest published error rate (i.e., highest fidelity) of all commercially available B-family DNA polymerases[36,37]. The manufacturer's protocol was modified to incorporate with-bead enzymatic and cleanup steps using Agencourt AMPure XP beads (Beckman-Coulter)[38]. Ligation was performed for 16 h at 16 °C using 100-fold molar excess of indexed Illumina TruSeq adapters. Single-step size selection was performed by adding 40 μL (0.8×) of PEG buffer to enrich for ligated DNA fragments. The ligated fragments were then amplified using 500 nM Illumina backbone oligonucleotides and 4–9 PCR cycles, depending on input DNA mass. Library purity and concentration was assessed by spectrophotometer (NanoDrop 2000) and qPCR (KAPA Biosystems), respectively. Fragment length was determined on a 2100 Bioanalyzer using the DNA 1000 Kit (Agilent).

Library design for hybrid selection. Hybrid selection was performed with a custom SeqCap EZ Choice Library (Roche NimbleGen). This library was designed through the NimbleDesign portal (v1.2.R1) using genome build hg19 NCBI Build 37.1/GRCh37 and with Maximum Close Matches set to 1. Input genomic regions were selected according to the most frequently mutated genes and exons in NSCLC and were chosen to iteratively maximize the number of mutations per tumor while minimizing selector size. These regions were identified from the COSMIC database, TCGA and other published sources as described in the **Supplementary Methods**. Final selector coordinates are provided in **Supplementary Table 1**.

Hybrid selection and next-generation sequencing. NimbleGen SeqCap EZ Choice was used according to the manufacturer's protocol with modifications. Between 9 and 12 indexed Illumina libraries were included in a single capture hybridization. Following hybrid selection, the captured DNA fragments were amplified with 12 to 14 cycles of PCR using 1× KAPA HiFi Hot Start Ready Mix and 2 μM Illumina backbone oligonucleotides in four to six separate 50-μL reactions. The reactions were then pooled and processed with the QIAquick PCR Purification Kit (Qiagen). Multiplexed libraries were sequenced using 100-bp paired-end runs on an Illumina HiSeq 2000. Raw sequencing data have been deposited in the Sequence Read Archive under accession number SRP040228.

Mapping and quality control. Paired-end reads were mapped to the hg19 reference genome with BWA 0.6.2 (default parameters)[39] and sorted and indexed with SAMtools[40]. Quality control (QC) was assessed using a custom Perl script to collect a variety of statistics, including mapping characteristics, read quality and selector on-target rate (i.e., number of unique reads that intersect the selector space divided by all aligned reads), generated respectively by SAMtools flagstat, FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and BEDTools coverageBed[41]. Plots of fragment length distribution and sequence depth and coverage were automatically generated for visual QC assessment. To mitigate the impact of sequencing errors, analyses not involving fusions were restricted to properly paired reads, and only bases with Phred quality scores ≥30 (≤0.1% probability of a sequencing error) were further analyzed.

Detection thresholds. Two dilution series were performed to assess the linearity and accuracy of CAPP-Seq for quantitating ctDNA. In one experiment, shorn genomic DNA from a NSCLC cell line (HCC78) was spiked into circulating DNA from a healthy individual, and in a second experiment, shorn genomic DNA from one NSCLC cell line (NCI-H3122) was spiked into shorn genomic DNA from a second NSCLC line (HCC78). A total of 32 ng DNA was used for library construction. Following mapping and quality control, homozygous reporters were identified as alleles unique to each sample with at least 20× sequencing depth and an allelic fraction >80%. Fourteen such reporters were identified between HCC78 genomic DNA and plasma DNA (**Fig. 2g,h**), whereas 24 reporters were found between NCI-H3122 and HCC78 genomic DNA (**Supplementary Fig. 5**).

Bioinformatics pipeline. Details of bioinformatics methods are supplied in the **Supplementary Methods**. Briefly, for detection of SNVs and indels, we employed VarScan 2 (ref. 42) with strict postprocessing filters to improve variant call confidence, and for fusion identification and breakpoint characterization, we used an algorithm called FACTERA (**Supplementary Methods**). To quantify tumor burden in plasma DNA, allele frequencies of reporter SNVs and indels were assessed using the output of SAMtools mpileup[40], and fusions, if detected, were enumerated with FACTERA.

Statistical analyses. The NSCLC selector was validated *in silico* using an independent cohort of lung adenocarcinomas[20] (**Fig. 1c**). To assess statistical significance, we analyzed the same cohort using 10,000 random selectors sampled from the exome, each with an identical size distribution to the CAPP-Seq NSCLC selector. The performance of random selectors had a normal distribution, and *P* values were calculated accordingly. Of note, all identified somatic lesions were considered in this analysis.

Related to **Figure 1d**, the probability (*P*) of recovering at least two reads of a single mutant allele in plasma for a given depth and detection limit was modeled by a binomial distribution. Given *P*, the probability of detecting all identified tumor mutations in plasma (for example, median of 4 for CAPP-Seq) was modeled by a geometric distribution. Estimates are based on 250 million 100-bp paired-end reads per lane (for example, using an Illumina HiSeq 2000 platform). Moreover, an on-target rate of 60% was assumed for CAPP-Seq and WES.

To evaluate the impact of reporter number on tumor burden estimates, we performed Monte Carlo sampling (1,000×), varying the number of reporters available {1,2,…*n*} in two spiking experiments (**Fig. 2g–i** and **Supplementary Fig. 4**).

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To assess the significance of tumor burden estimates in plasma DNA using SNVs, we compared patient-specific SNV frequencies to the null distribution of selector-wide background alleles. Indels were analyzed separately using mutation-specific background rates and *Z*-score statistics. Fusion breakpoints were considered significant when present with >0 read support due to their ultralow false detection rate.

For each patient, we calculated a ctDNA detection index (akin to a false-positive rate) based on *P* value integration from his or her array of reporters (**Table 1** and **Supplementary Table 4**). Specifically, for cases where only a single reporter type was present in a patient's tumor, the corresponding *P* value was used. If SNV and indel reporters were detected and if each independently had a *P* value <0.1, we combined their respective *P* values using Fisher's method[43]. Otherwise, given the prioritization of SNVs in the selector design, the SNV *P* value was used. If a fusion breakpoint identified in a tumor sample (i.e., involving *ROS1*, *ALK* or *RET*) was recovered in plasma DNA from the same patient, it trumped all other mutation types, and its *P* value (~0) was used. If a fusion detected in the tumor was not found in corresponding plasma (potentially owing to hybridization inefficiency; **Supplementary Methods**), the *P* value for any remaining mutation type(s) was used. The ctDNA detection index was considered significant if the metric was ≤0.05 (approximate false-positive rate ≤5%), the threshold that maximized CAPP-Seq sensitivity and specificity in ROC analyses (determined by Euclidean distance to a perfect classifier; i.e., a true-positive rate equal to 1 and a false-positive rate equal to 0; **Figs. 3** and **4**, **Table 1** and **Supplementary Table 4**).

We evaluated CAPP-Seq performance in a blinded fashion by masking all patient identifying information, including disease stage, circulating DNA time point, treatment, etc. We then applied our ctDNA detection index across the entire grid of deidentified plasma DNA samples (13 patient-specific sets of somatic reporters across 40 plasma samples, or 520 pairs). To calculate sensitivity and specificity, we 'unblinded' ourselves and grouped patient samples into cancer-positive (i.e., cancer was present in the patient's body), cancer-negative (i.e., patient was cured) or cancer-unknown (i.e., insufficient data to determine true classification) categories (**Fig. 3a**,**b** and **Supplementary Fig. 6**). ROC analyses and significance estimates were performed using GraphPad Prism 6.

Additional details are presented in the **Supplementary Methods**.

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Published OnlineFirst April 24, 2018; DOI: 10.1158/1078-0432.CCR-17-3831

Precision Medicine and Imaging

Clinical Cancer Research

Validation of a Plasma-Based Comprehensive Cancer Genotyping Assay Utilizing Orthogonal Tissue- and Plasma-Based Methodologies

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Abstract

Purpose: To analytically and clinically validate a circulating cell-free tumor DNA sequencing test for comprehensive tumor genotyping and demonstrate its clinical feasibility.

Experimental Design: Analytic validation was conducted according to established principles and guidelines. Blood-to-blood clinical validation comprised blinded external comparison with clinical droplet digital PCR across 222 consecutive biomarker-positive clinical samples. Blood-to-tissue clinical validation comprised comparison of digital sequencing calls to those documented in the medical record of 543 consecutive lung cancer patients. Clinical experience was reported from 10,593 consecutive clinical samples.

Results: Digital sequencing technology enabled variant detection down to 0.02% to 0.04% allelic fraction/2.12 copies with ≤0.3%/2.24–2.76 copies 95% limits of detection while maintaining high specificity [prevalence-adjusted positive predictive values (PPV) >98%]. Clinical validation using orthogonal plasma- and tissue-based clinical genotyping across >750 patients demonstrated high accuracy and specificity [positive percent agreement (PPAs) and negative percent agreement (NPAs) >99% and PPVs 92%–100%]. Clinical use in 10,593 advanced adult solid tumor patients demonstrated high feasibility (>99.6% technical success rate) and clinical sensitivity (85.9%), with high potential actionability (16.7% with FDA-approved on-label treatment options; 72.0% with treatment or trial recommendations), particularly in non–small cell lung cancer, where 34.5% of patient samples comprised a directly targetable standard-of-care biomarker.

Conclusions: High concordance with orthogonal clinical plasma- and tissue-based genotyping methods supports the clinical accuracy of digital sequencing across all four types of targetable genomic alterations. Digital sequencing's clinical applicability is further supported by high rates of technical success and biomarker target discovery. *Clin Cancer Res; 24(15); 3539–49. ©2018 AACR.*

Introduction

Modern systemic cancer therapy is evolving away from cytotoxic polychemotherapy to embrace novel therapeutics targeting molecular characteristics specific to individual patients' tumors that deliver superior clinical outcomes at reduced toxicity and overall cost (1, 2). Indeed, precision oncology is the preferred standard of care in National Comprehensive Cancer Network (NCCN) treatment guidelines (3) for advanced non–small cell lung cancer (NSCLC), colorectal cancer, melanoma, breast cancer, gastric and gastroesophageal adenocarcinoma, and gastrointestinal stromal tumors and is rapidly maturing in multiple other indications. Because of the increasing diversity of targeted therapies and associated molecular biomarkers, serial testing via traditional singleplex methods, such as PCR and FISH, has become impractical in some diseases (4). Congruently, the 2017 NCCN NSCLC practice guidelines explicitly recommend testing via "broad molecular profiling" by next-generation sequencing (NGS) methods validated to detect multiple variants simultaneously across all four genomic variant classes [base substitutions (single-nucleotide variants (SNV)), insertions/deletions (indel), gene amplifications (copy number alterations (CNA)), and gene rearrangements (fusions); ref. 4].

Even when broad molecular profiling is adopted, however, precision oncology has historically remained dependent on tissue-based biomarker assessment, which limits patient benefit in four primary ways. First, tissue sampling, whether by core needle biopsy, surgical biopsy, or fine-needle approaches, is necessarily invasive and burdened with the morbidity, mortality, costs, and delays associated with these procedures (5–7). Second, tissue sampling has variable but substantial failure rates due to patient ineligibility, procedural/sampling failure,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-17-3831

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Published OnlineFirst April 24, 2018; DOI: 10.1158/1078-0432.CCR-17-3831

Translational Relevance

Plasma-based comprehensive tumor genotyping expands access to standard-of-care precision oncology to patients previously ineligible due to barriers associated with tissue sampling. Despite this and the current clinical use of multiple such "liquid biopsies," comprehensive, structured validation studies of relevant patient populations of meaningful size using validated orthogonal comparator methods are lacking, which leaves clinicians without the knowledge necessary to properly vet and interpret available options. Here, we describe definitive analytic and clinical validation studies of a comprehensive tumor-derived cell-free DNA sequencing assay for clinical genotyping of advanced solid tumors and report clinical experience applying this technology in the clinical care of 10,593 consecutive patients. These data establish this technology as a clinically effective, accurate tumor genotyping alternative for patients for whom invasive tissue acquisition procedures are infeasible.

or material insufficiency, which range from 20% to more than 50% in second-line advanced lung cancer patients (8–10). Third, tissue sampling is unavailable for a substantial minority of patients due to medical contraindication, patient unwillingness, and/or logistical concerns; congruently, extant data on genotyping rates demonstrate only moderate adherence to practice guidelines in the first line (65%–75%) and poor adherence in the second and later (<25%; refs. 11, 12). Fourth, tissue sampling is necessarily limited to a single, small tumor focus, which may not accurately represent all clinically relevant biomarkers due to spatial and temporal heterogeneity (13–16). Taken together, these data describe critical and avoidable missed treatment opportunities for patients with advanced solid tumors (8, 14, 17).

The advent of tumor genotyping from peripheral blood ("liquid biopsy") via circulating cell-free tumor DNA (ctDNA) obviates these limitations and expands access to standard-of-care precision oncology to patients previously ineligible due to barriers associated with tissue sampling (4, 8, 9, 18–22). Despite this, comprehensive, structured validation studies of relevant patient populations of meaningful size using validated orthogonal comparator methods are lacking, with existing studies failing to adhere to established comparison study design principles (23). Here, we describe the analytic (technical characterization using controlled and often contrived samples) and clinical (characterization using real-world patient samples, contexts, and comparators) validation of a comprehensive ctDNA sequencing assay for clinical genotyping of advanced solid tumors, including large-scale comparisons with orthogonal clinical plasma- and tissue-genotyping methods. We further report our experience applying this technology in the clinical care of 10,593 consecutive patients in our Clinical Laboratory Improvement Amendment (CLIA)–certified, College of American Pathologists (CAP)–accredited, New York State Department of Health–approved laboratory, which represents the largest published dataset of clinical liquid biopsy experience to date.

Materials and Methods

Blood draw, shipment, and plasma isolation

All patient samples were collected and processed in accordance with the Guardant360 Clinical Blood Collection Kit instructions (Guardant Health, Inc.). Briefly, 2 × 10 mL of peripheral venous blood was collected in Streck Cell-Free DNA BCT (Streck, Inc.), packaged into a provided padded thermal insulation block within a secondary biohazard containment barrier, and shipped overnight to Guardant Health at ambient temperature. Although samples were accepted up to 7 days after blood collection, the median draw-to-receipt interval was 1.43 days. Upon receipt, samples were accessioned and proceeded immediately to plasma isolation (median accessioning-to-isolation interval 17 minutes). To isolate plasma, whole blood was centrifuged (1,600 × *g* for 10 minutes at 10°C), and the resulting supernatant was clarified by additional centrifugation (3,220 × *g* for 10 minutes at 10°C). Clarified plasma was transferred to a fresh tube and stored at 2°C for immediate extraction or stored at −80°C.

cfDNA extraction, library preparation, and sequencing

All cell-free DNA (cfDNA) extraction, processing, and sequencing was performed in a CLIA-certified, CAP-accredited laboratory (Guardant Health, Inc.) as described previously (24). Briefly, cfDNA was extracted from the entire plasma aliquot prepared from a single 10-mL tube described above (QIAamp Circulating Nucleic Acid Kit, Qiagen, Inc.), which yielded a median of 48.5 ng of cfDNA (range, 2–1,050 ng). Five to 30 ng of extracted cfDNA (66% of samples processed with 30 ng input) was labeled with nonrandom oligonucleotide barcodes (IDT, Inc.) and used to prepare sequencing libraries, which were then enriched by hybrid capture (Agilent Technologies, Inc.), pooled, and sequenced by paired-end synthesis (NextSeq 500 and/or HiSeq 2500, Illumina, Inc.). Contrived analytic samples were generated using similarly prepared cfDNA from healthy donors (AllCells, Inc.) and cfDNA isolated as above from the culture supernatant of model cell lines (ATCC, Inc.; Sigma, Inc.) and serially size-selected using Agencourt AMPure XP beads (Beckman Coulter, Inc.) until no detectable gDNA remained. Separate sequencing controls are utilized for SNVs and CNAs/fusions/indels (CFI). The SNV control comprises a mixture of healthy donor cfDNA pooled to target germline SNVs to 0.5%, 2.5%, and 6% allelic fraction. The CFI control comprises cell lines with known CNAs, fusions, and indels diluted into healthy donor cfDNA.

Bioinformatics analysis and variant detection

All variant detection analyses were performed using the locked clinical Guardant360 bioinformatics pipeline and reported unaltered by *post hoc* analyses. All decision thresholds were determined using independent training cohorts, locked, and applied prospectively to all validation and clinical samples. As described previously (24), base call files generated by Illumina's RTA software (v2.12) were demultiplexed using bcl2fastq (v2.19) and processed with a custom pipeline for molecule barcode detection, sequencing adapter trimming, and base quality trimming (discarding bases below Q20 at the ends of the reads). Processed reads were then aligned to hg19 using BWA-MEM (arXiv:1303.3997v2) and used to build double-stranded consensus representations of original unique cfDNA molecules using both inferred molecular barcodes and read start/stop positions. SNVs were detected by comparing read and consensus molecule characteristics to

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sequencing platform- and position-specific reference error noise profiles determined independently for each position in the panel by sequencing a training set of 62 healthy donors on both the NextSeq 500 and HiSeq 2500. Observed positional SNV error profiles were used to define calling cutoffs for SNV detection with respect to the number and characteristics of variant molecules, which differed by position but were most commonly $\geq$2 unique molecules, which in an average sample (~5,000 unique molecule coverage) corresponds to a detection limit of approximately 0.04% variant allele fraction (VAF). Indel detection used two methods. For short (<50–70 bp) indels, a generative background noise model was constructed to account for PCR artifacts arising frequently in homopolymeric or repetitive contexts, allowing for strand-specific and late PCR errors. Detection was then determined by the likelihood ratio score for observed feature-weighted variant molecule support versus background noise distribution. Detection of indels $\gg$50 bp relies on secondary analysis of soft-clipped reads using methods described in the fusion section below and is only performed to detect specific genomic events (e.g., *MET* exon 14–skipping deletions). Reporting thresholds were event-specific as determined by performance in training samples but were most commonly at least one unique molecule for clinically actionable indels, which in an average sample corresponds to a detection limit of approximately 0.02% VAF. Fusion events were detected by merging overlapping paired-end reads to form a representation of the sequenced cfDNA molecule, which was then, aligned, mapped to initial unique cfDNA molecules based on molecular barcoding and alignment information, including soft clipping. Soft-clipped reads were analyzed using directionality and breakpoint proximity to identify clusters of molecules representing candidate fusion events, which were then used to construct fused references against which reads soft-clipped by the aligner on the first pass were realigned. Specific reporting thresholds were determined by retrospective and training set analyses but were generally $\geq$2 unique postrealignment molecules meeting quality requirements, which in an average sample corresponds to a detection limit of approximately 0.04% VAF. To detect CNAs, probe-level unique molecule coverage was normalized for overall unique molecule throughput, probe efficiency, GC content, and signal saturation and robustly summarized at the gene level. CNA determinations were based on training set–established decision thresholds for both absolute copy number deviation from per-sample diploid baseline and deviation from the baseline variation of probe-level normalized signal in the context of background variation within each sample's own diploid baseline. Per-sample relative tumor burden was determined by normalization to the mutational burden expected for tumor type and ctDNA fraction and reported as a *Z*-score.

Analytic validation approach

In the analytic validation of the assay, we adhered to established CLIA, Nex-StoCT Working Group, and Association of Molecular Pathologists/CAP guidance regarding performance characteristics and validation principles. To define clinically meaningful performance, the entire assay workflow was mapped against possible clinical sample contexts, points and mechanisms of possible failure or inaccuracy identified, and validation studies designed to systematically examine each of these critical areas as per Clinical and Laboratory Standards (CLSI) guidance EP23-A. As such, the validation cohort comprises broad variant diversity and is heavily enriched for alterations with low VAFs and/or near decision boundaries. All studies were conducted using contrived materials and subsequently verified using patient samples, both of which were themselves validated using orthogonal methods and reference materials wherever possible. In addition, all studies were conducted at standard input amounts using the locked clinical Guardant360 bioinformatics pipeline and results reported unaltered by *post hoc* analyses. All decision thresholds were predetermined using independent training cohorts, locked, and applied prospectively to all validation and clinical samples.

To assess sensitivity for sequence variants (SNVs, indels, and fusions), cfDNA samples with orthogonally confirmed (e.g., ddPCR, tissue genotyping, exome sequencing, published characterization studies) variants were titrated into donor cfDNA targeting $\geq$5 prespecified VAFs at both standard (30 ng) and minimum (5 ng) cfDNA inputs. Multiple replicates of each titration point were then processed and used to calculate the detection probability for both clinically actionable and background variants and define the 95% limit of detection (LOD) both empirically and by probit regression. CNAs, in contrast, are not detectable at the level of individual molecules and are instead defined by statistical overrepresentation of reference sequences in the cfDNA. As such, we utilized the CLSI "Classical Approach" (CLSI EP17-A2) to statistically determine copy number LOD based on variation observed in titration series replicates and normal samples by calculating the limit where estimated copy number and *Z*-score values jointly have a 95% probability of exceeding the decision threshold.

Accuracy studies comprised orthogonally verified contrived and clinical samples representing the entire reportable range; however, cohorts were heavily enriched for variants at near and below the LOD and in unusual sequence contexts. Prevalence-adjusted PPV as a function of allelic frequency was defined as follows: $PPV_i = \frac{(F_i^C - F_i^N)}{F_i^C}$, where F_i^C is per-sample frequency of somatic calls in clinical samples within VAF range i, and F_i^N is per-sample frequency of false positive calls in known negative samples in allelic range i. A total of 2,585 previously analyzed consecutive clinical samples were used to define somatic alteration frequency by VAF. Frequency of false positive calls was defined using a set of 408 validation runs (324 LOD pools and 84 healthy cfDNA donor runs across both NextSeq and HiSeq instruments) for which truth was orthogonally defined. Analytic specificity was determined by analysis of pooled samples at high dilution that had been orthogonally defined at high VAF. Precision was determined utilizing in-process positive controls from the clinical sequencing workflow and pooled clinical samples.

Orthogonal validation methods

For in-house assay ddPCR, sequence variant analyses were performed using the Bio-Rad droplet digital PCR (ddPCR) platform with QX200 droplet reader (Bio-Rad, Inc.). SNVs and indels were quantified using variant-specific PCR primer pairs and wild-type or mutant-specific fluorescent hydrolysis probes (Bio-Rad, Inc., IDT, Inc.). Copy number determination was assessed using a compendium of multiplexed PCR assays against reference genes in combination with target-specific PCR assays. Copy number was determined from the average of the reference gene signal relative to the total target signal. For external clinical ddPCR studies, deidentified clinical samples were submitted to the Dana-Farber Cancer Institute Translational Research Laboratory

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for ddPCR testing as described previously (25). The variant test was determined from available assays (*EGFR* L858R, exon 19 deletions, and T790M; *KRAS* G12X/G13D; *BRAF* V600X; *ESR1* D538G and Y537C; and *PIK3CA* H1047R) by the Laboratory's choice based on a provided clinical diagnosis; no variant or result information was provided. For external tissue concordance, tissue genotyping was conducted as part of routine clinical care and comprised a variety of methods based on PCR, sequencing, ISH, or IHC.

All studies were conducted in accordance with recognized ethical guidelines (e.g., Declaration of Helsinki, CIOMS, Belmont Report, U.S. Common Rule) and with a waiver of patient consent by an Institutional Review Board–approved protocol.

Results

Assay design

The assay, Guardant360, utilizes NGS-based digital sequencing (DS; ref. 24) to comprehensively profile 73 cancer-related genes (Supplementary Fig. S1) comprising both therapeutically relevant biomarkers, including those for all approved and many investigational drugs and clinical trials, and cancer-specific biomarkers that may be used to establish both ctDNA presence and quantity. Briefly, cfDNA is extracted from stabilized whole blood, labeled at high efficiency with nonrandom oligonucleotide adapters ("molecular barcodes"), and used to prepare sequencing libraries, which are then enriched using hybrid capture and sequenced (Fig. 1). Sequencing reads are then used to reconstruct each individual cfDNA molecule present in the original patient sample with high-fidelity using proprietary double-stranded consensus sequence representation.

The combination of high-efficiency (80%–90%) input molecule labeling, optimized assay conditions, and hybridization probe design allows DS to recover 60%–85% of all input molecules postalignment (Fig. 2A), depending on cfDNA input, across the entire range of G:C content (Fig. 2B) with highly uniform coverage (13% quartile-based coefficient of variation with >96% of exonic panel positions covered above 0.2× median panel coverage; Fig. 2C). In addition, DS's *in silico* molecule reconstruction suppresses substitution and indel errors intrinsic to NGS workflows by more than three orders of magnitude relative to standard sequencing approaches, from approximately $10^{-3.5}$ to $10^{-6.5}$ per base (Fig. 2D and E), which allows accurate variant calling down to VAFs of 0.01%/1 molecule for indels and 0.04%/2 molecules for SNVs and fusions (summarized in Supplementary Table S1). Critically, >50% of molecules are reconstructed from both strands of the original cfDNA molecule, greatly increasing consensus sequence fidelity and specificity over other previously published approaches (26–32), which integrate both strands in only 12% or fewer of recovered unique molecules.

SNV detection performance

To validate the sensitivity and accuracy of SNV detection, donor cfDNA comprising 43 nonredundant germline variants defined by orthogonal exome analysis of leukocyte genomic DNA (gDNA) and patient sample cfDNA comprising 12 actionable somatic SNVs confirmed by ddPCR was titrated to 5 prespecified VAFs in triplicate bracketing the estimated 95% limit of detection (LOD_{95}) in 753 total observations. An additional patient cfDNA pool comprising 67 diluted germline variants and 22 actionable somatic SNVs was also processed at VAFs representative of clinical somatic variant prevalence. Within the titration series, the LOD_{95}

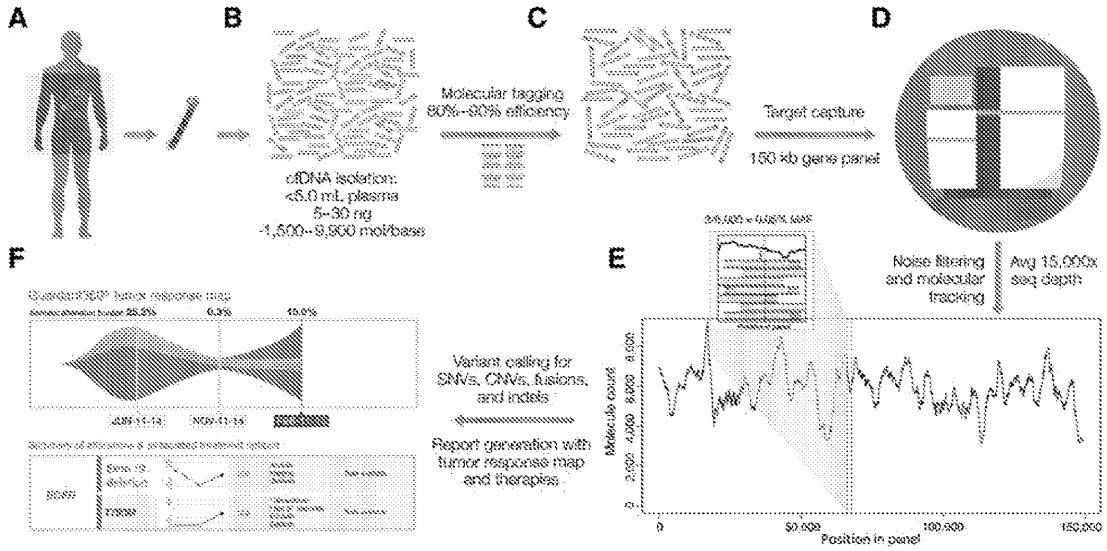


Figure 1.

DS–based ctDNA assay workflow. **A–C,** cfDNA is extracted from stabilized peripheral whole blood (**A**), labeled with oligonucleotide barcodes at high efficiency (**B**), and up to 30 ng is used for library preparation (**C**). **D,** Sequencing libraries are enriched using hybrid capture and sequenced to an average depth of approximately 15,000×. **E,** Individual unique input molecules are then bioinformatically reconstructed using barcode and sequence data to suppress analytic error modes. **F,** Somatic variants are deconvoluted from germline and reported by clinical priority with both treatment and clinical trial annotations.

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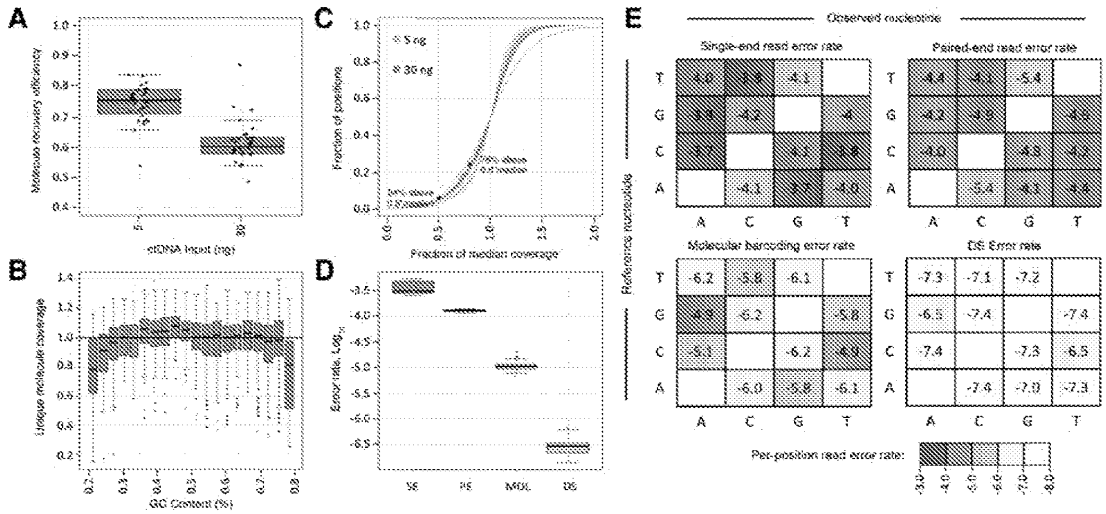


Figure 2.

Technical assay performance characteristics. **A,** Unique molecule recovery postsequencing as a function of input. **B** and **C,** Unique molecule coverage as a function of GC content (**B**) and per position as a fraction of median coverage (**C**). Total intrinsic (**D**) and specific base substitution (**E**) error rates after each layer of DS error correction for 42 healthy donor cfDNA samples. SE, single-end reads; PE, paired-end reads; MOL, molecular barcoding.

was determined empirically to be 0.3% (0.20% by probit) for actionable and slightly above 0.4% (94% detection rate, 0.34% by probit) for variants of uncertain significance (Fig. 3A; Supplementary Fig. S2A). Overall, detection above the LOD_{95} was high (122/122, 100%), with high prevalence–adjusted PPV (99.2%). Importantly, even at VAFs below the LOD_{95} (0.05%–0.25%), SNV detection remained moderate (35/55, 63.8%) and accuracy high (PPV 96.3%; Fig. 3C), which is critical for ctDNA analyses where many clinically relevant alterations are present at very low levels. When variants associated with clonal hematopoiesis (identified by reproducible detection on replicate analysis within and between sequencing platforms) were excluded, PPV across the validation dataset rose to 99.96%. Similarly, in 667 serial replicates of a well-defined cfDNA reference material used as an in-process sequencing control, only 20 of 42,496 total variants (VAF, 0.5%–6%) detected were unexpected, corresponding to an analytic PPV of 99.95% and a per-sample specificity of 97%. Importantly, in all studies, PPV of clinically actionable SNVs was 100% both above and below the LOD_{95}. SNV detection also maintained quantitative accuracy at VAFs near the LOD_{95}; across 871 observations between 0.05% and 1.0%, the correlation between observed and expected VAFs was high (r^2 = 0.84, y = 1.06; Supplementary Fig. S3A).

Indel detection performance

To validate indel detection performance, samples comprising 3 ddPCR-confirmed driver and 13 tumor suppressor indels across 11 genes were diluted to create an 8-point titration series comprising 200 near-LOD observations. The LOD_{95} was established empirically at 0.2% and 0.7% for driver and tumor suppressor indels, respectively (0.11% and 0.43% by probit; Fig. 3A; Supplementary Fig. S2B). These data were supplemented with samples comprising 25 additional indels across 10 genes verified by either literature (cell lines), tissue genotyping (patient samples), or ddPCR (both) to determine an indel identification accuracy of 100% (34/34). Prevalence-adjusted PPV for all indel calls was estimated at 98.2% both above and below the LOD_{95}. PPV for actionable indels remained 100% across the entire reportable range of VAFs. Per-sample analytic specificity using donor cfDNA was 100% (42/42) and 98% (49/50) for undiluted and pooled donors and 99.7% (665/667) in sequencing control material described above.

Fusion detection performance

To assess fusion detection performance, samples comprising 4 cell line- and 8 patient-derived cfDNA fusions were titrated to generate 93 near-LOD observations. The LOD_{95} was determined empirically to be 0.2% (0.22% by probit; Fig. 3A; Supplementary Fig. S2C). Including additional cell lines and patient samples, analytic accuracy was determined to be 95% (20/21 unique fusions), and analytic PPV was 100% both above and below the LOD_{95}. Per-sample analytic specificity using donor cfDNA was 100% for undiluted (42/42) and pooled (50/50) donors and in sequencing control material (667/667).

CNA detection performance

To validate CNA detection performance, cell line cfDNA harboring known amplifications of *ERBB2*, *MET*, *EGFR*, *MYC*, *CCND1*, and *CCNE1* was used to bracket the expected LOD_{95} in 120 near-LOD observations. LOD_{95} estimates constructed for each gene independently ranged from 2.24 copies (*ERBB2*) to 2.76 (*CCND2*, the smallest gene on the panel), median 2.44 (Fig. 3A; Supplementary Fig. S2D). Using multiplex-baselined ddPCR, we validated CNA accuracy in 14 cell lines comprising 43 amplifications (3–74 copies, median 4) in 10 genes. Importantly, DS results correlated closely with copy number results derived

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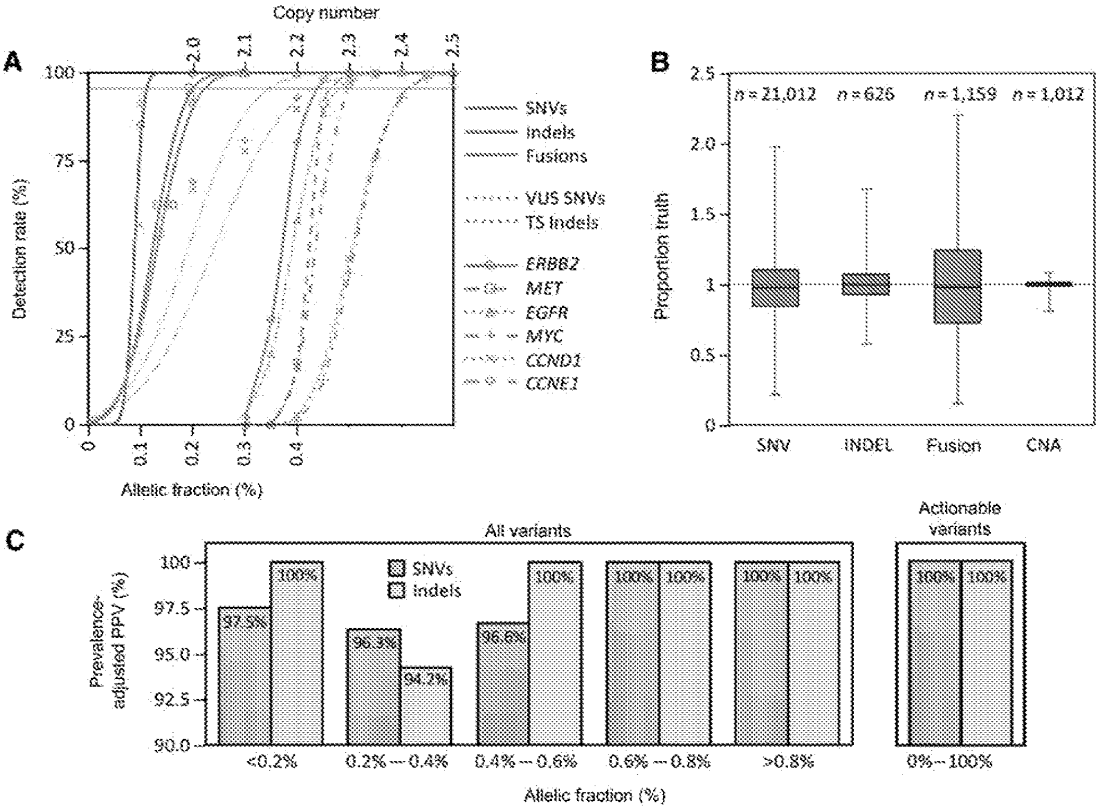


Figure 3.

Somatic variant detection performance. **A,** Probit plot of detection probability as a function of allelic fraction for oncogenic driver and VUS SNVs, oncogene and tumor suppressor indels, oncogenic fusions, and 6 different gene amplifications. Sequence variants correspond to the lower *x*-axis (allelic fraction); gene amplifications correspond to the upper *x*-axis (copy number). Empirical observations include matched color. Horizontal gray line, 95% detection probability. **B,** Normalized allelic fraction or copy number across 375 consecutive SNVs and copy number-fusion-indel control runs. Number of observations across all replicates indicated. Horizontal gray line indicates allelic fraction truth as indicated by orthogonal testing. **C,** PPV by allelic fraction for all SNVs and indels and those associated with a therapy or clinical trial adjusted by variant prevalence in a training set of 5,285 consecutive clinical samples. VUS, variant of uncertain significance; TS, tumor suppressor.

from both ddPCR ($r^2 = 0.98$, slope = 1.03) and Cancer Cell Line Encyclopedia microarray measurements ($r^2 = 0.84$, slope = 1.16; Supplementary Fig. S3B and S3C). As no unexpected positives were observed in the 257 characterized samples within the validation dataset, CNA detection PPV was determined to be 100%. Using well-characterized cfDNA CNA-specific sequencing control material, analytic PPV was measured to be 98.3% (226/230). Analytic specificity using healthy donor cfDNA was 100% for undiluted (42/42) and pooled (50/50) donors and 99.9% (3,712/3,716) in CNA-specific sequencing control material.

Variant detection precision

To determine run-to-run precision, we analyzed positive sequencing controls included in each batch of clinical samples. Across 375 consecutive runs of one SNV control lot, precision of exonic SNVs was 99.9% for 6% VAF (n = 749/750), 99.5% for 2.5% VAF (n = 8,583/8,625), and 97.6% for 0.5% VAF (n = 6,954/7,125). Across those same 375 runs, which comprised multiple copy number-fusion-indel control lots, indel (unique n = 4, 1.0%–7.5% VAF), fusion (unique n = 6, 1.0%–2.0% VAF), and copy number precision (unique n = 6, 2.4–5.2 copies) was 100%. Quantitative VAF and copy number measurements were consistent over more than 370 runs (Fig. 3B; Supplementary Fig. S4) with 95% of SNVs, indels, fusions, and CNAs within 32.5%, 26.1%, 73.6%, and 5.2% of targeted levels, respectively.

Clinical validation studies

To validate clinical accuracy, we submitted 222 consecutive *EGFR*, *KRAS*, *BRAF*, and/or *ESR1*-positive NSCLC, colorectal cancer, or breast cancer specimens received for clinical testing to the Dana-Farber Cancer Institute (Boston, MA) for blinded analysis using a highly validated clinical ddPCR panel (Fig. 4A and B; Supplementary Table S2; ref. 25). DS and ddPCR were concordant for 269 positive calls [0.1%–94% VAF, positive percent agreement

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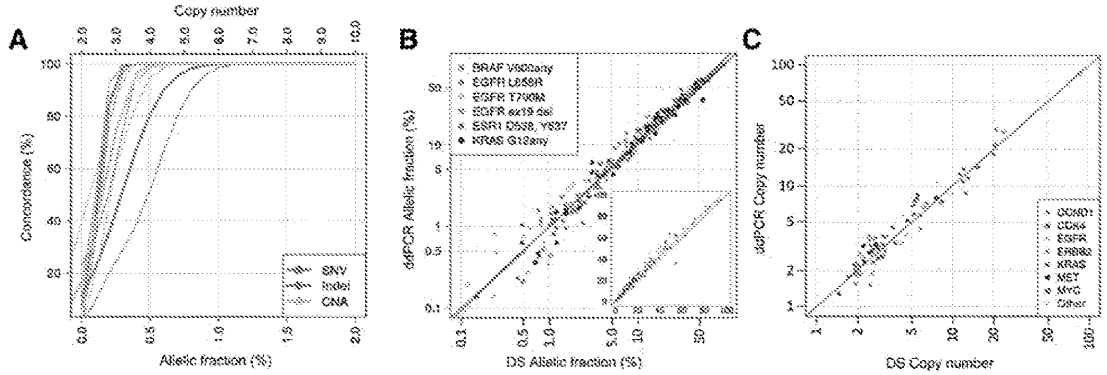


Figure 4.

Concordance between DS and digital PCR in clinical samples. **A,** Positive concordance between DS and clinical ddPCR for 222 SNVs (blue) and 55 indels (gray) by VAF and 70 CNAs (green) by copy number plotted as probit model estimates (bold lines) with 95% confidence intervals (thin lines). **B** and **C,** Quantitative correlation with linear regression for SNVs and indels (**B**) and CNAs (**C**).

(PPA) 99.6%] and 308 negative [negative percent agreement (NPA) 97.8%]. Only a single variant, *KRAS* G12C, was detected by ddPCR but not DS (at 0.18%, below DS's LOD_{95}), while DS detected 3 *EGFR* exon 19 deletions and 4 T790M SNVs (0.10%–0.39% VAF) at or below ddPCR's reportable range (25), most probably due to DS's greater per reaction cfDNA input (DS interrogates up to 30 ng in a single reaction, whereas ddPCR spreads this across 3 separate reactions). Importantly, all discordant results occurred below either one or both assays' LOD and/or reportable range. Moreover, clinical follow-up of such samples demonstrated support for positive calls in 6 of 8 discordant cases (Supplementary Table S3).

We similarly assessed the accuracy of copy number determination relative to ddPCR in 70 samples with detectable CNAs in 8 genes (copy numbers 2.33–48.0) and 49 samples without. Together, ddPCR and DS calls were concordant for 63 of 70 positive calls and all 49 negative (PPA 90%, NPA 100%; Fig. 4A). Importantly, all 7 discordant calls were near the decision threshold of one or both assay platforms; outside of this region, concordance was 100%.

To validate quantitative accuracy, we compared DS's VAFs and copy numbers to ddPCR measurements (Fig. 4B and C). As expected, SNV and indel VAFs and CNA copy numbers were highly correlated (r^2 = 0.994, 0.995, and 0.943 respectively; y = 0.944, 0.922, and 0.980, respectively).

To assess ctDNA-tissue genotyping concordance, we conducted a blinded, IRB-approved retrospective review of samples received for clinical testing, distinct from the studies above, in which we extracted external molecular testing results from submitted pathology reports and determined whether variants identified by DS were present by established clinical genotyping methods. From an initial cohort of 6,948 consecutive NSCLC patients, we identified 543 for whom DS calls could be compared with external tissue genotyping results. Across seven genomic biomarkers (*EGFR, ALK, ROS1, RET, BRAF, MET,* and *KRAS*) clinically relevant for primary therapy selection, the positive concordance was 92% to 100% (Fig. 5). Importantly, *post hoc* clinical follow-up of the 3 patients positive for *ALK* fusions by DS but negative by FISH revealed that these patients had not only been treated with crizotinib irrespective of the diagnostic discordance but had also responded clinically as assessed by clinician judgment (33).

Clinical experience

Validation studies, such as those described above, are critical to establish technical performance metrics and verify clinical performance; however, clinical utility is predicated on the ability to apply a test in real-world clinical care and return results in a reliable and timely manner. Moreover, test performance on large numbers of patients can often provide insights into test accuracy than smaller, controlled validation studies. To these ends,

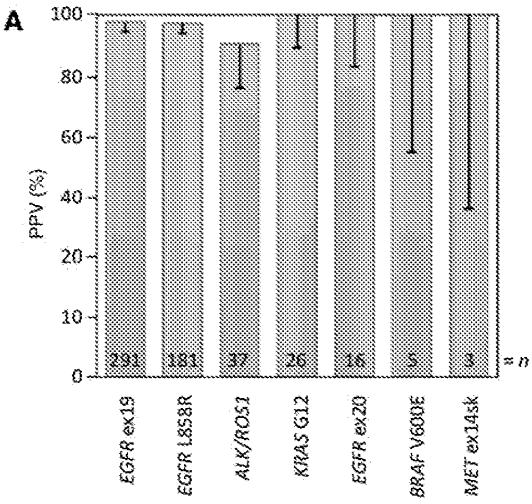


Figure 5.

Confirmation of DS variants detected in clinical samples by tissue genotyping. **A,** PPV of DS calls relative to tissue genotyping results derived from patient medical records across 543 clinical samples.

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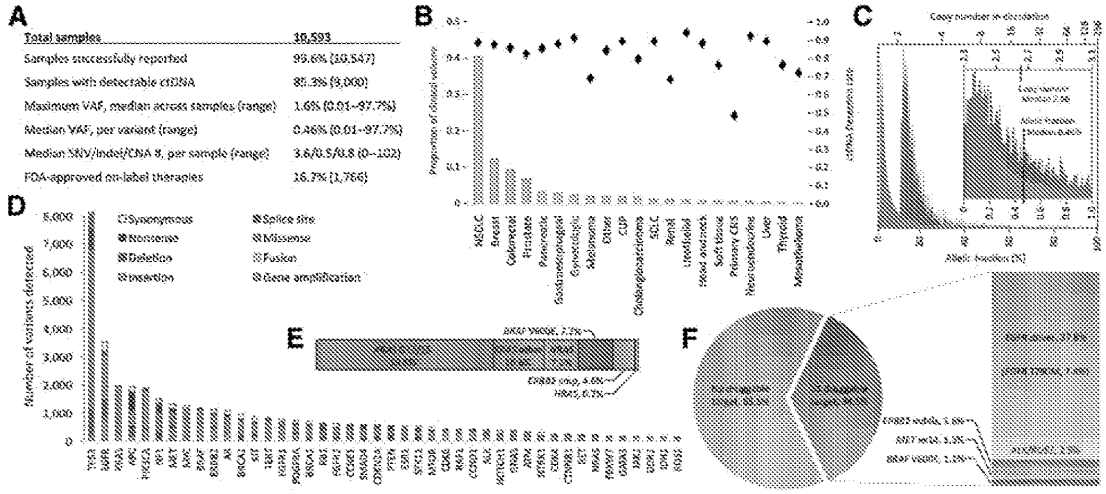


Figure 6.
Clinical DS of 10,585 advanced cancer patients. **A,** Cohort-descriptive statistics. **B,** Proportion of clinical samples by tumor type. Diamonds indicate ctDNA detection rate for each tumor type. **C,** Distribution of tumor ctDNA burden as represented by the maximum VAF or copy number of a somatic variant in a given sample. **D,** Somatic alteration prevalence by variant type and gene. **E,** Prevalence of alterations associated resistance to anti-EGFR mAb therapy. **F,** Targetable alteration prevalence in 4,521 consecutive nonsquamous NSCLC patients. CUP, carcinoma of unknown primary; SCLC, small-cell lung cancer; CNS, central nervous system.

we analyzed consecutive clinical samples submitted to our CLIA-certified, CAP-accredited clinical laboratory. As our test is intended only for systemic therapy selection, all samples are derived from patients with advanced tumors. Within the study period, 10,593 samples were submitted with 10,585 (99.9%) comprising sufficient cfDNA for analysis and 10,547 successfully reported (99.6% analytic success rate; Fig. 6A). ctDNA detection rate overall was high (85.9%), predominantly driven by NSCLC (87.7%), colorectal (85.0%), and breast (86.8%); however, certain tumors, for example, primary central nervous system malignancies, were less likely to comprise detectable ctDNA, due to smaller typical tumor volumes and anatomic considerations, such as the blood–brain barrier (Fig. 6B).

Alteration prevalence was markedly enriched at low VAFs (median VAF 0.46%) even when restricted to targetable driver mutations (Fig. 6C), emphasizing the need for highly sensitive diagnostics. At least one variant with therapeutic or clinical trial connotations was identified in 72.0% (7,591/10,547) of samples, and although individual patients typically comprised fewer than two per sample, the superset comprised 3,479 unique alterations, underscoring the importance of broad genomic profiling. Congruent with previous reports of high ctDNA–gDNA concordance (8, 9, 24, 34, 35), per-variant and VAF-normalized relative tumor mutation burden landscape analyses closely match those of previous reports (Fig. 6D; Supplementary Fig. S5; refs. 36–38). At least one of 155 total unique variants associated with FDA-approved on-label therapies was identified in 16.7% (1,766/10,547) of samples, and at least one of 133 total unique variants associated with resistance to these approved drugs was identified in 13.9% (1,467/10,547). In colorectal cancer patients, 66.7% (548/819) comprised alterations associated with resistance to anti-EGFR antibody therapy, only 54.9% (301/548) of which were detectable by common (codons 12/13) *KRAS* testing (Fig. 6E). In 4,521 NSCLC patients, 34.5% comprised at least one alteration directly targetable with standard-of-care therapeutics (Fig. 6F), compatible with previously reported alteration frequencies (36, 37).

Discussion

Precision oncology is associated with improved outcomes, reduced adverse effect profiles, and reduced overall cost; however, its application is limited by reliance on tissue-based biomarker assessment, which shackles it to invasive biopsy procedures associated with substantial cost, morbidity, mortality, and failure rates. In contrast, ctDNA-based liquid biopsies decouple biomarker assessment from tissue, enabling tumor-specific genotyping using safe, inexpensive, and near-painless peripheral blood sampling. Although promising, successful implementation of liquid biopsy is fraught with challenges including ctDNA's short fragment lengths (~165 bp), scant material (median 48 ng/10 mL whole blood vs. micrograms in typical tissue sections), low tumor representation (VAFs typically 10- to 100-fold lower than tissue), and absent reference materials and methods.

We have developed an NGS-based ctDNA diagnostic that overcomes these challenges to provide highly accurate tumor-specific genotyping for all guideline-recommended advanced solid tumor somatic biomarkers from a single peripheral blood draw. This test demonstrates exceptional sensitivity ($LOD_{95} \leq 0.3\%$ for SNVs, indels, and fusions and near 2.2 copies for CNAs), while maintaining high PPV (>98%) even in sample cohorts enriched for alterations at or below the applicable LOD. Specificity and precision are similarly high, allowing accurate variant identification

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Published OnlineFirst April 24, 2018; DOI: 10.1158/1078-0432.CCR-17-3831

as low as 0.02% to 0.04%, which is critical for clinical ctDNA analysis as many relevant alterations are present at very low levels. Performance was verified in 349 clinical samples using internal and blinded external comparison to clinical ddPCR, which demonstrated very high qualitative and quantitative concordance. Analysis of 543 matched tissue genotyping results demonstrated high PPVs relative to standard-of-care tissue diagnostics. Of note, clinical response to targeted therapy in the three *ALK* fusion ctDNA-positive/tissue-negative patients is congruent with previous reports (8, 39, 40), highlighting a weakness of method comparison with imperfect reference methodologies as compared with the gold standard of clinical response. Clinical sensitivity was not assessable in this tissue genotyping comparison study; however, other studies have reported PPAs relative to tissue varying substantially between 52% and 100% (8, 9, 21–24, 34, 41–44), with most between 70% and 90%, depending on the specific biomarker, tissue test, clinical indication, overlap of comparator panels and capabilities, and time elapsed between tissue and plasma sampling (21, 23).

When used clinically for 10,593 consecutive patients, DS demonstrated robust technical success (>99.6%), ctDNA detection (85.9%), and rapid result delivery (median time from sample receipt to report, 7 days). Although at least one variant with therapeutic or clinical trial connotations was identified in 72.0% of samples, these comprised 3,479 unique alterations in aggregate, underscoring the importance of broad genomic profiling. Importantly, 16.7% of samples harbored variants with on-label treatment recommendations, whereas 13.9% harbored variants predictive of resistance to the same. Underscoring the importance of highly sensitive diagnostics, >50% of alterations were detected below 0.5% VAF.

These studies comprise the largest published ddPCR and tissue concordance series and demonstrate near-perfect concordance for driver alterations. Similarly, mutational prevalence and mutational burden analyses mirror findings from previous tissue-based reports (36–38). These high concordances strongly support existing evidence demonstrating the validity and utility of DS-guided treatment, which includes 17 clinical outcome studies for genomically targeted therapies (8, 9, 19–22, 34, 42–48) and TMB-based (49) immunotherapy. In contrast to limited/hotspot ctDNA panels, Guardant360's 73-gene design also allows interpretation of negative findings (required for effective biopsy prevention) and early detection of immunotherapy resistance (50, 51) through inclusion of all major driver oncogenes and tumor suppressors.

These data demonstrate applicability of ctDNA-based genotyping to treatment paradigms based on tissue genotyping; however, ctDNA has the potential to not only match but to surpass tissue genotyping in four important ways: First, ctDNA, as a circulating analyte, is derived from the entire tumor burden and thus has potential to capture tumor heterogeneity that tissue-based methods, which sample only a small focus, cannot achieve. Indeed, plasma-based detection of *ALK* fusions not present in focal tissue biopsies in the tissue comparison study above and those patients' subsequent therapeutic responses illustrates the potential impact of tumor heterogeneity. Second, the noninvasive nature of liquid biopsy enables longitudinal analyses not feasible with invasive tissue biopsies. Testing for acquired therapy resistance is a current application of longitudinal analysis with proven therapeutic relevance; however, other potential applications include therapy monitoring, early prediction of immunotherapy efficacy, and disease surveillance. Third, as ctDNA production is proportional to tumor growth, the fastest-growing tumor clones shed the most material, which makes ctDNA enriched for the most biologically aggressive, and likely most clinically relevant, tumor cells. Fourth, the quantitative accuracy of ctDNA-based genotyping enables discrimination of dominant versus subclonal alterations over both time and space and may potentially allow more accurate therapeutic targeting (13–16).

In summary, we present the analytic and clinical validation of a highly accurate noninvasive option for comprehensive tumor genomic profiling of advanced solid tumors, including high concordance to both orthogonal clinical plasma- and tissue-based genotyping assays, and present real-life clinical use data in >10,000 advanced cancer patients, demonstrating its feasibility and value in delivering both established and novel benefits of precision oncology to patients for whom tissue is unavailable.

Disclosure of Potential Conflicts of Interest

J.I. Odegaard is an employee of Guardant Health. J.J. Vincent is an employee of and holds ownership interest (including patents) in Guardant Health. S.R. Fairclough holds ownership interest (including patents) in Guardant Health. O.A. Zill holds ownership interest (including patents) in Guardant Health. C.E. Lee holds ownership interest (including patents) in Guardant Health. L.A. Kiedrowski holds ownership interest (including patents) in Guardant Health. C.P. Paweletz reports receiving commercial research grants from Guardant Health and is a consultant/advisory board member for AstraZeneca, Bio-Rad, and DropWorks, Inc. R. B. Lanman is an employee of and holds ownership interest (including patents) in Guardant Health. D.I. Chudova is an employee of and holds ownership interest (including patents) in Guardant Health. A. Talasaz is an employee of and holds ownership interest (including patents) in Guardant Health. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: J.I. Odegaard, S. Mortimer, H. Eltoukhy, D.I. Chudova

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Received December 26, 2017; revised March 15, 2018; accepted April 20, 2018; published first April 24, 2018.

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EX1002.853

Published OnlineFirst April 24, 2018; DOI: 10.1158/1078-0432.CCR-17-3831

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Published OnlineFirst April 24, 2018; DOI: 10.1158/1078-0432.CCR-17-3831

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/601,168	10/14/2019	Helmy ELTOUKHY	42534-708.303	1052

115823 7590 02/18/2020

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EXAMINER	
HORLICK, KENNETH R	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
02/18/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents@guardanthealth.com
patentdocket@wsgr.com

Office Action Summary	Application No. 16/601,168	Applicant(s) ELTOUKHY et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 31-60 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 31-60 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 10/14/19 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date See Continuation Sheet.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Continuation of Attachment(s) 2) Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date: 1/14/20; 1/14/20; 1/14/20; 1/14/20; 1/14/20; 1/14/20; 1/14/20; 1/14/20.

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

NON-PRIOR ART REJECTIONS

2. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., In re Berg, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); In re Goodman, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); In re Longi, 759 F.2d 887, 225 USPQ645 (Fed. Cir. 1985); In re Van Ornum, 686 F.2d 937, 214 USPQ761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ619 (CCPA 1970); In re Thorington, 418 F.2d 528, 163 USPQ644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(l) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

3. Claims 31-60 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-33 of U.S. Patent No. 9,902,992. Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the patented claims are related as genus-species. That is, the steps of the instant claims are included within the steps of the patent claims.

4. Claims 31-60 are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 10 of U.S. Patent No. 9,920,366. Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the patented claim are related as genus-species. That is, the steps of the instant claims are included within the steps of the patented claim.

5. Claims 57-60 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 42 of copending Application No. 15/892,178 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the copending claim are related as genus-species. That is, the steps of the instant claims are included within the steps of the copending claim.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claim has not in fact been patented.

PRIOR ART REJECTION

6. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

7. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

Claims 31-60 are rejected under 35 U.S.C. 103 as being unpatentable over Schmitt et al. (US 9,752,188; effective filing date 3/20/12) in view of Sacko et al. (US 2010/0264331).

Independent claim 31 is drawn to a method comprising: ligating adapters comprising barcodes to double-stranded cell-free DNA molecules, using at least a 10X molar excess of adapters, wherein at least 20% of DNA molecules are tagged; amplifying tagged molecules; selectively enriching for tagged molecules with target regions associated with cancer; sequencing enriched tagged strands to generate sequence reads; mapping sequence reads; grouping sequence reads into families; and detecting one or

more somatic genetic variants. Independent claim 57 is drawn to a similar but broader method which lacks the above steps of enriching, mapping, and grouping.

Schmitt et al. discloses a method comprising: tagging double-stranded DNA molecules with a set of duplex tags comprising barcodes; selectively enriching (amplifying) for tagged strands that map to a genetic locus; sequencing enriched tagged strands to generate sequence reads; grouping sequence reads into families; collapsing sequence reads into consensus sequence reads, and detecting mutations (somatic genetic variants). See columns 5-30, especially column 20, line 39 to column 21, line 25.

Schmitt et al. does not disclose wherein at least a 10X molar excess of adapters is used such that at least 20% of molecules are tagged, nor wherein the double-stranded DNA is cell-free DNA.

Sacko et al. discloses that cell-free DNA from blood of cancer patients is associated with microsatellite mutations and instabilities, which are one type of somatic genetic variant (see paragraph 0005).

One of ordinary skill in the art would have been motivated to modify the method of Schmitt et al. by using at least a 10X molar excess of adapters such that at least 20% of molecules are tagged because this would have merely involved routine optimization of known-important reaction parameters, which as well established in U.S. patent practice does not support unobviousness (see M.P.E.P. 2144.05). The skilled artisan would have been motivated to modify the method of Schmitt et al. by using cell-free DNA because Sacko et al. disclosed that cell-free DNA may contain somatic genetic variants associated with cancers. It is submitted that the further limitations of the dependent claims also fall within the category of routine optimization of known-important reaction parameters. Thus, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the application was filed to carry out the claimed methods.

CONCLUSION

8. No claims are free of the prior art.

9. Any inquiry concerning this communication or earlier communications from the examiner should be directed to KENNETH R HORLICK whose telephone number is (571)272-0784. The examiner can normally be reached on Mon. - Thurs. 8:30 - 6:30.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see https://ppair-my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

02/12/20

/KENNETH R HORLICK/
Primary Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 16/601,168	Applicant(s)/Patent Under Reexamination ELTOUKHY et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	Page 1 of 1

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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20100264331-A1	10-2010	Sacko; Mory	G01N21/6428	250/459.1
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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

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First Named Inventor	Helmy ELTOUKHY
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.303

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	38	Guardant Health vs. FMI 1st Amended Answer to Second Amended Complaint dated May 6, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	
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	40	Guardant Health vs. FMI Invalidity Exhibit A-1, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	41	Guardant Health vs. FMI Invalidity Exhibit A-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	42	Guardant Health vs. FMI Invalidity Exhibit A-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
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	45	Guardant Health vs. FMI Invalidity Exhibit B-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
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	50	Guardant Health vs. FMI Invalidity Exhibit C-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	

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	Examiner Name	
	Attorney Docket Number	42534-708.303

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-14
Name/Print	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Application Number	16601168
Filing Date	2019-10-14
First Named Inventor	Helmy ELTOUKHY
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.303

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Name/Print	Timothy A. Hott	Registration Number	67740

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Name/Print	Timothy A. Hott	Registration Number	67740

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1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

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8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

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(Not for submission under 37 CFR 1.99)

Application Number	16601168
Filing Date	2019-10-14
First Named Inventor	Helmy ELTOUKHY
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.303

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	1	Guardant Health vs. FMI Invalidity Exhibit C-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	2	Guardant Health vs. FMI Invalidity Exhibit C-4, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	3	Guardant Health vs. FMI Invalidity Exhibit C-5, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	4	Guardant Health vs. FMI Invalidity Exhibit D-1, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	5	Guardant Health vs. FMI Invalidity Exhibit D-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	6	Guardant Health vs. FMI Invalidity Exhibit D-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	7	Guardant Health vs. FMI Invalidity Exhibit D-5, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	8	Guardant Health vs. FMI Second Amended Complaint dated March 6, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	9	Guardant Health vs. FMI Suppl Invalidity References dated February 15, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	
	10	Guardant Health vs. FMI Supplemental Invalidity Contentions dated March 29, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	
	11	Guardant Health vs. PGDx Amended Answer to Second Amended Complaint dated April 30, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	

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	12	Guardant Health vs. PGDx Invalidity Claim Chart A, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	13	Guardant Health vs. PGDx Invalidity Claim Chart B, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	14	Guardant Health vs. PGDx Invalidity Claim Chart C, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	15	Guardant Health vs. PGDx Invalidity Claim Chart D, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	16	Guardant Health vs. PGDx Invalidity Claim Chart E, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	17	Guardant Health vs. PGDx Invalidity Claim Chart F, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	18	Guardant Health vs. PGDx Invalidity Claim Chart G, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	19	Guardant Health vs. PGDx Invalidity Claim Chart H, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	20	Guardant Health vs. PGDx Invalidity Claim Chart I, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	21	Guardant Health vs. PGDx Invalidity Claim Chart J, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	22	Guardant Health vs. PGDx Invalidity Claim Chart K, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	

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	23	Guardant Health vs. PGDx Invalidity Claim Chart L, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
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	25	Guardant Health vs. PGDx Second Amended Complaint dated March 23, 2018 (C.A. No. 17-cv-1623-LPS-CJB)	
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	50	Illumina "Preparing Samples for Paired-End Sequencing (2008)	

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Examiner Signature	/KENNETH R HORLICK/	Date Considered	02/11/2020

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-14
Name/Print	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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	1	INGOLIA, et al. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science. 2009 Apr 10;324(5924):218-23. Epub 2009 Feb 12.	
	2	Instructions for Norit Rapid DNA Ligation Kit (November 6, 2004)	
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	11	IPR2018-00130, Petition for Inter Partes Review of U.S. Patent 9,598,731, dated November 7, 2018	

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	12	IPR2019-00130, Decision Denying Inter Partes Review U.S. 9,598,731, issued June 5, 2019	
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	14	IPR2019-00634, Decision Instituting Inter Partes Review U.S.9,840,743, issued August 19, 2019	
	15	IPR2019-00634, Petition for Inter Partes Review of U.S. Patent 9,840,743, dated February 1, 2019	
	16	IPR2019-00636 & 637, Exhibit 3001 Memo for Inter Partes Review of U.S. Patent 9,902,992, dated July 1, 2019	
	17	IPR2019-00636 & 637, Order - Conduct of the Proceedings for Inter Partes Review of U.S. Patent 9,902,992, dated July 22, 2019	
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The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-14
Name/Print	Timothy A. Hott	Registration Number	67740

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	8	Notice of allowance dated 06/15/2017 for US Application No.15/076,565.	
	9	Notice of allowance dated 06/19/2014 for US Application No. 12/969,581.	
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	15	Notice of allowance dated 10/03/2017 for US Application No. 15/076,565.	
	16	Notice of allowance dated 10/25/2017 for US Application No. 14/861,989	
	17	Notice of allowance dated 12/28/2017 for US Application No. 14/861,989.	
	18	Office Action dated 02/09/2017 for U.S. Patent Application No. 15/076,565.	
	19	Office action dated 05/13/2019 for US Application No. 15/669,779.	
	20	Office action dated 05/20/2016 for US Application No. 14/855,301.	
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	24	Office action dated 06/12/2017 for US Application No. 15/492,659.	
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	37	Opposition Brief dated August 7, 2018 for Japanese Opposition No. 2018-700659 to JP Patent 6275145	☒
	38	Opposition Decision dated January 15, 2019 for Japanese Opposition No. 2018-700659 to JP Patent 6275145	☒
	39	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Foundation Medicine Inc.	☐
	40	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Grunecker Patent-und Rechtsanwalte PartG mbB	☐
	41	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Personal Genome Diagnostics, Inc.	☐
	42	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Strawman Limited	☐
	43	Opposition Form and Statement to EP3087204 filed November 14, 2018	☐
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	16601168
Filing Date	2019-10-14
First Named Inventor	Helmy ELTOUKHY
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.303

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	49	DAINES, et al. High-throughput multiplex sequencing to discover copy number variants in Drosophila. Genetics. 2009 Aug;182(4):935-41. doi: 10.1534/genetics.109.103218. Epub 2009 Jun 15.	
	50	D'ANTONI, et al. Rapid quantitative analysis using a single molecule counting approach. Anal Biochem. 2006 May 1;352(1):97-109. Epub 2006 Feb 10.	

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/KENNETH R HORLICK/	Date Considered	02/11/2020

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16601168
	Filing Date	2019-10-14
	First Named Inventor	Helmy ELTOUKHY
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.303

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-14
Name/Print	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/672,267	01/07/2020	AmirAli TALASAZ	42534-708.304	3448

115823 7590 03/03/2020

Wilson Sonsini Goodrich & Rosati / Guardant Health
650 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
HORLICK, KENNETH R	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
03/03/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents@guardanthealth.com
patentdocket@wsgr.com

Office Action Summary	Application No. 16/672,267	Applicant(s) TALASAZ et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 31-60 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 31-60 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 11/1/19 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date See Continuation Sheet.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Continuation of Attachment(s) 2) Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date: 12/6/19; 12/6/19; 12/6/19; 12/6/19; 12/6/19; 12/6/19; 12/6/19; 12/6/19

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

NON-PRIOR ART REJECTIONS

2. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., In re Berg, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); In re Goodman, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); In re Longi, 759 F.2d 887, 225 USPQ645 (Fed. Cir. 1985); In re Van Ornum, 686 F.2d 937, 214 USPQ761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ619 (CCPA 1970); In re Thorington, 418 F.2d 528, 163 USPQ644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(l) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-l.jsp.

3. Claims 31-60 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-33 of U.S. Patent No. 9,902,992. Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the patented claims are related as genus-species. That is, the steps of the instant claims are included within the steps of the patent claims.

4. Claims 31-60 are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1-21 of U.S. Patent No. 9,920,366. Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the patented claim are related as genus-species. That is, the steps of the instant claims are included within the steps of the patented claim.

5. Claims 31-60 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 31-60 of copending Application No. 16/601,168 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the copending claims are related as genus-species. That is, the steps of the instant claims are included within the steps of the copending claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

6. Claims 31-60 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 31-60 of copending Application No. 16/714,579 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the copending claims are related as obvious species-genus. That is, the 'more than a 30X molar excess' of the instant claims is a clearly suggested species within the genus 'at least a 10X molar excess' of the copending claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

PRIOR ART REJECTION

7. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

8. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

Claims 31-60 are rejected under 35 U.S.C. 103 as being unpatentable over Schmitt et al. (US 9,752,188; effective filing date 3/20/12) in view of Deciu et al. (US 2013/0288244; effective filing date 3/14/13), and further in view of Sacko et al. (US 2010/0264331).

These claims are drawn to methods comprising: ligating barcodes to cell-free nucleic acid molecules, using more than a 30X molar excess of barcodes relative to cell-free nucleic acid molecules, wherein at least 20% of said molecules are attached to barcodes; amplifying tagged molecules; and selectively enriching tagged molecules for genomic regions of interest.

Schmitt et al. discloses a method comprising: tagging DNA molecules with a set of tags comprising barcodes; and selectively enriching (amplifying) for tagged strands that map to a region of interest. See columns 5-30, especially column 20, line 39 to column 21, line 25.

Schmitt et al. does not disclose wherein more than a 30X molar excess of adapters/barcodes is used such that at least 20% of molecules are tagged, nor wherein the DNA is cell-free DNA.

Deciu et al. discloses the desirability to optimize ligation of adaptors to both ends of a polynucleotide (see paragraph [1152]).

Sacko et al. discloses detecting cell-free DNA from blood of cancer patients because it comprises DNA having microsatellite mutations and instabilities, which are useful in diagnostics (see paragraph 0005).

One of ordinary skill in the art would have been motivated to modify the method of Schmitt et al. by using more than a 30X molar excess of adapters/barcodes such that at least 20% of nucleic acid molecules are tagged because this would have merely involved routine optimization of known-important reaction parameters, which as well established in U.S. patent practice does not support unobviousness (see M.P.E.P. 2144.05). This is supported by Deciu et al., which discloses the desirability to optimize ligation of adaptors to both ends of a polynucleotide in paragraph [1152]. Ligation reactions were textbook-level subject matter for which the important parameters, such as enzyme and polynucleotide component concentrations, were unarguably known. It is submitted that the further limitations of the dependent claims also fall within the category of routine optimization of known-important reaction parameters. The skilled artisan would have been motivated to modify the method of Schmitt et al. by using cell-free DNA because Sacko et al. disclosed that cell-free DNA may contain somatic genetic variants associated with cancers, and is thus useful in diagnostics. It would have been *prima facie* obvious to one of ordinary skill in the art at the time the application was filed to carry out the claimed methods.

CONCLUSION

9. No claims are free of the prior art.

10. Any inquiry concerning this communication or earlier communications from the examiner should be directed to KENNETH R HORLICK whose telephone number is (571)272-0784. The examiner can normally be reached on Mon. - Thurs. 8:30 - 6:30.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see https://ppair-my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

02/27/20

/KENNETH R HORLICK/
Primary Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 16/672,267	Applicant(s)/Patent Under Reexamination TALASAZ et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20100264331-A1	10-2010	Sacko; Mory	G01N21/6428	250/459.1
*	B	US-20130288244-A1	10-2013	DECIU; Cosmin	G16B20/00	435/6.11
	C					
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
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NON-PATENT DOCUMENTS

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	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16672267
Filing Date	2019-11-01
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.304

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16672267
	Filing Date	2019-11-01
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.304

	1	NAWROZ, et al. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med. 1996 Sep;2 (9):1035-7.	
	2	NEWMAN, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014 May;20(5):548-54. doi: 10.1038/nm.3519. Epub 2014 Apr 6.	
	3	NG, S.B., et al., "Targeted capture and massively parallel sequencing of 12 human exomes," Nature 2009, 461(7261), 272-276	
	4	NIELSEN, R. et al. "Genotype and SNP calling from next-generation sequencing data" Nature Reviews Genetics (2011) 12(6):443-451	
	5	NORD, et al. Accurate and exact CNV identification from targeted high-throughput sequence data. BMC Genomics. 2011 Apr 12;12:184.	
	6	Notice of Allowance dated 01/31/2017 for U.S. Patent Application No. 14/712,754.	
	7	Notice of allowance dated 03/21/2014 for US Application No. 12/969,581.	
	8	Notice of allowance dated 06/15/2017 for US Application No.15/076,565.	
	9	Notice of allowance dated 06/19/2014 for US Application No. 12/969,581.	
	10	Notice of allowance dated 08/01/2017 for US Application No. 15/492,659	
	11	Notice of allowance dated 08/04/2017 for US Application No.15/467,570	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16672267
	Filing Date	2019-11-01
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.304

	12	Notice of allowance dated 08/22/2014 for US Application No. 12/969,581.	
	13	Notice of allowance dated 08/29/2017 for US Application No. 15/492,659	
	14	Notice of allowance dated 09/11/2017 for US Application No. 15/076,565	
	15	Notice of allowance dated 10/03/2017 for US Application No. 15/076,565.	
	16	Notice of allowance dated 10/25/2017 for US Application No. 14/861,989	
	17	Notice of allowance dated 12/28/2017 for US Application No. 14/861,989.	
	18	Office Action dated 02/09/2017 for U.S. Patent Application No. 15/076,565.	
	19	Office action dated 05/13/2019 for US Application No. 15/669,779.	
	20	Office action dated 05/20/2016 for US Application No. 14/855,301.	
	21	Office action dated 05/31/2016 for US Application No. 14/712,754.	
	22	Office action dated 06/01/2017 for US Application No. 15/467,570	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	2019-11-01
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.304

	23	Office action dated 06/03/2016 for US Application No. 14/861,989.	
	24	Office action dated 06/12/2017 for US Application No. 15/492,659.	
	25	Office action dated 07/18/2017 for US Application No. 14/861,989	
	26	Office Action dated 07/30/2019 for U.S. Application No. 16/283,635.	
	27	Office action dated 08/06/2019 for US Application No. 14/855,301.	
	28	Office action dated 10/03/2013 for US Application No. 12/969,581.	
	29	Office Action dated 10/12/2016 for U.S. Patent Application No. 14/712,754.	
	30	Office action dated 10/20/2017 for US Application No. 14/425,189.	
	31	Office Action dated 11/21/2016 for U.S. Patent Application No. 14/855,301.	
	32	Office action dated 12/04/2015 for US Application No. 14/712,754.	
	33	Office action dated 12/07/2017 for US Application No. 14/855,301	

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	34	Office Action dated 12/09/2016 for U.S. Patent Application No. 14/861,989.	
	35	Office action dated 12/21/2018 for US Application No. 15/978,848.	
	36	OGINO, et al. Quantification of PCR bias caused by a single nucleotide polymorphism in SMN gene dosage analysis. J Mol Diagn. 2002 Nov;4(4):185-90.	
	37	Opposition Brief dated August 7, 2018 for Japanese Opposition No. 2018-700659 to JP Patent 6275145	☒
	38	Opposition Decision dated January 15, 2019 for Japanese Opposition No. 2018-700659 to JP Patent 6275145	☒
	39	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Foundation Medicine Inc.	☐
	40	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Grunecker Patent-und Rechtsanwalte PartG mbB	☐
	41	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Personal Genome Diagnostics, Inc.	☐
	42	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Strawman Limited	☐
	43	Opposition Form and Statement to EP3087204 filed November 14, 2018	☐
	44	Pacific Biosciences. Template Preparation and Sequencing Guide. Publication date: 2014-10-14. Pacific Biosciences website http://www.pacificbiosciences.com/support/pubmap/documentation.html.	☐

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	45	PAN, et al. Loss of heterozygosity patterns provide fingerprints for genetic heterogeneity in multistep cancer progression of tobacco smoke-induced non-small cell lung cancer. Cancer Res. 2005 Mar 1;65(5):1664-9.	☐
	46	PARK, et al. Discovery of common Asian copy number variants using integrated high-resolution array CGH and massively parallel DNA sequencing. Nat Genet. 2010 May;42(5):400-5. doi: 10.1038/ng.555. Epub 2010 Apr 4.	☐
	47	PARKINSON, N.J. et al., "Preparation of high-quality next-generation sequencing libraries from picogram quantities of target DNA," Genome Res. 2012, 22(1), 125-133	☐
	48	PEL, J. et al. "Duplex proximity sequencing (pro-seq): A method to improve DNA sequencing accuracy without the cost of molecular barcoding redundancy" bioRxiv (2017) https://doi.org/10.1101/163444	☐
	49	PERAKIS, S. et al. "Advances in Circulating Tumor DNA Analysis" Adv Clin Chem (2017) pp 1-81	☐
	50	PIHLAK, et al. Rapid genome sequencing with short universal tiling probes. Nature Biotechnology, 26: 676-684 (2008).	☐

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

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Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2019-12-06
Name/Print	Timothy A. Hott	Registration Number	67740

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	1	Guardant Health vs. FMI Invalidity Exhibit C-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	2	Guardant Health vs. FMI Invalidity Exhibit C-4, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	3	Guardant Health vs. FMI Invalidity Exhibit C-5, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	4	Guardant Health vs. FMI Invalidity Exhibit D-1, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	5	Guardant Health vs. FMI Invalidity Exhibit D-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	6	Guardant Health vs. FMI Invalidity Exhibit D-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	7	Guardant Health vs. FMI Invalidity Exhibit D-5, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	8	Guardant Health vs. FMI Second Amended Complaint dated March 6, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	9	Guardant Health vs. FMI Suppl Invalidity References dated February 15, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	
	10	Guardant Health vs. FMI Supplemental Invalidity Contentions dated March 29, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	
	11	Guardant Health vs. PGDx Amended Answer to Second Amended Complaint dated April 30, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	

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	12	Guardant Health vs. PGDx Invalidity Claim Chart A, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	13	Guardant Health vs. PGDx Invalidity Claim Chart B, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	14	Guardant Health vs. PGDx Invalidity Claim Chart C, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	15	Guardant Health vs. PGDx Invalidity Claim Chart D, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	16	Guardant Health vs. PGDx Invalidity Claim Chart E, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	17	Guardant Health vs. PGDx Invalidity Claim Chart F, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	18	Guardant Health vs. PGDx Invalidity Claim Chart G, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
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	37	HIBI, et al. Molecular detection of genetic alterations in the serum of colorectal cancer patients. Cancer Res. 1998 Apr 1;58(7):1405-7.	
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	50	Illumina "Preparing Samples for Paired-End Sequencing (2008)	

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First Named Inventor	AmirAli TALASAZ
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Examiner Name	
Attorney Docket Number	42534-708.304

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Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

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Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2019-12-06
Name/Print	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Name/Print	Timothy A. Hott	Registration Number	67740

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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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Application Number	16672267
Filing Date	2019-11-01
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.304

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	3	2013181170	WO	A1	2013-12-05	OTWINOWSKI et al.	Entire Document	

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	4	2013188471	WO	A2	2013-12-19	Faham et al.		
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	10	2014093330	WO	A1	2014-06-19	Raymond et al.		
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	13	2014151117	WO	A1	2014-09-25	Diehn et al.	Entire Document	
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	38	Guardant Health vs. FMI 1st Amended Answer to Second Amended Complaint dated May 6, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	
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	40	Guardant Health vs. FMI Invalidity Exhibit A-1, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
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Name/Print	Timothy A. Hott	Registration Number	67740

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3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16672267
Filing Date	2019-11-01
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.304

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	15	IPR2019-00634, Petition for Inter Partes Review of U.S. Patent 9,840,743, dated February 1, 2019	
	16	IPR2019-00636 & 637, Exhibit 3001 Memo for Inter Partes Review of U.S. Patent 9,902,992, dated July 1, 2019	
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	20	IPR2019-00637, Petition for Inter Partes Review of U.S. Patent 9,902,992, dated February 1, 2019	
	21	IPR2019-00652, Decision Granting Inter Partes Review U.S.9,834,822, issued August 19, 2019	
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If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/KENNETH R HORLICK/	Date Considered	02/26/2020

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.304

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2019-12-06
Name/Print	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/714,579	12/13/2019	AmirAli TALASAZ	42534-708.305	3111

115823 7590 03/03/2020

Wilson Sonsini Goodrich & Rosati / Guardant Health
650 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
HORLICK, KENNETH R	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
03/03/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents@guardanthealth.com
patentdocket@wsgr.com

Office Action Summary	Application No. 16/714,579	Applicant(s) TALASAZ et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 31-60 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 31-60 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 12/13/19 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date See Continuation Sheet.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Continuation of Attachment(s) 2) Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date: 1/16/20; 1/16/20; 1/16/20; 1/16/20; 1/16/20; 1/16/20; 1/16/20; 1/16/20

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

NON-PRIOR ART REJECTIONS

2. The following is a quotation of 35 U.S.C. 112(b):

(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 46 is rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention. This claim is confusing because of the language in line 1 'the wherein the'. Correction is required.

3. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., In re Berg, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); In re Goodman, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); In re Longi, 759 F.2d 887, 225 USPQ645 (Fed. Cir. 1985); In re Van Ornum, 686 F.2d 937, 214 USPQ761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ619 (CCPA 1970); In re Thorington, 418 F.2d 528, 163 USPQ644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided

the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(l) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-l.jsp.

4. Claims 31-60 is rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-33 of U.S. Patent No. 9,902,992. Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the patented claims are related as genus-species. That is, the step of the instant claim is included within the steps of the patent claims.

5. Claims 31-60 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-21 of U.S. Patent No. 9,920,366. Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the patented claims are related as genus-species. That is, the step of the instant claims is included within the steps of the patent claims.

6. Claims 31-60 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 31-53 of copending Application No. 15/892,178 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the copending claims are related as genus-species. That is, the step of the instant claims is included within the steps of the copending claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

7. Claims 31-60 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 31-60 of copending Application No. 16/601,168 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the copending claims are related as genus-species. That is, the step of the instant claims is included within the steps of the copending claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

8. Claims 31-60 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 31-60 of copending Application No. 16/672,267 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims and the copending claims are related as genus-species. That is, the step of the instant claims is included within the steps of the copending claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

PRIOR ART REJECTION

9. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of

the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

10. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

Claims 31-60 are rejected under 35 U.S.C. 103 as being unpatentable over Schmitt et al. (US 9,752,188; effective filing date 3/20/12) in view of Deciu et al. (US 2013/0288244; effective filing date 3/14/13).

Claims 31-48 are drawn to a method comprising: tagging polynucleotide molecules with a set of library adaptors comprising barcodes, wherein at least a 10X molar excess of library adaptors is used relative to polynucleotide molecules, and at least 20% of molecules are tagged at both ends. Claims 49-60 are drawn to such a method, additionally comprising: amplification, enrichment, determining sequence, and computer analysis.

Schmitt et al. discloses a method comprising: tagging double-stranded DNA molecules with a set of duplex tags comprising barcodes; selectively enriching (amplifying) for tagged strands that map to a genetic locus; sequencing enriched tagged strands to generate sequence reads; and grouping

sequence reads into families and collapsing sequence reads into consensus sequence reads using computer analysis. See columns 5-30, especially column 20, line 39 to column 21, line 25.

Schmitt et al. does not disclose wherein at least a 10X molar excess of library adaptors is used relative to polynucleotide molecules, and wherein at least 20% of molecules are tagged at both ends.

One of ordinary skill in the art would have been motivated to modify the method of Schmitt et al. by using conditions such that at least a 10X molar excess of library adaptors is used relative to polynucleotide molecules, and at least 20% of molecules are tagged, because this would have merely involved routine optimization of known-important reaction parameters, which as well established in U.S. patent practice does not support unobviousness (see M.P.E.P. 2144.05). This is supported by Deciu et al., which discloses the desirability to optimize ligation of adaptors to both ends of a polynucleotide in paragraph [1152]. Ligation reactions were textbook-level subject matter for which the important parameters, such as enzyme and polynucleotide component concentrations, were unarguably known. It is submitted that the further limitations of the dependent claims also fall within the category of routine optimization of known-important reaction parameters. Thus, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the application was filed to carry out the claimed methods.

CONCLUSION

11. No claims are free of the prior art.

12. Any inquiry concerning this communication or earlier communications from the examiner should be directed to KENNETH R HORLICK whose telephone number is (571)272-0784. The examiner can normally be reached on Mon. - Thurs. 8:30 - 6:30.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see https://ppair-my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

02/27/20

/KENNETH R HORLICK/
Primary Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 16/714,579	Applicant(s)/Patent Under Reexamination TALASAZ et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	Page 1 of 1

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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16714579
Filing Date	2019-12-13
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.305

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Examiner Signature	/KENNETH R HORLICK/	Date Considered	02/26/2020

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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	Examiner Name	
	Attorney Docket Number	42534-708.305

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-16
Name/Print	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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	Filing Date	2019-12-13
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.305

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-16
Name/Print	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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	7	Notice of allowance dated 03/21/2014 for US Application No. 12/969,581.	
	8	Notice of allowance dated 06/15/2017 for US Application No.15/076,565.	
	9	Notice of allowance dated 06/19/2014 for US Application No. 12/969,581.	
	10	Notice of allowance dated 08/01/2017 for US Application No. 15/492,659	
	11	Notice of allowance dated 08/04/2017 for US Application No.15/467,570	

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	12	Notice of allowance dated 08/22/2014 for US Application No. 12/969,581.	
	13	Notice of allowance dated 08/29/2017 for US Application No. 15/492,659	
	14	Notice of allowance dated 09/11/2017 for US Application No. 15/076,565	
	15	Notice of allowance dated 10/03/2017 for US Application No. 15/076,565.	
	16	Notice of allowance dated 10/25/2017 for US Application No. 14/861,989	
	17	Notice of allowance dated 12/28/2017 for US Application No. 14/861,989.	
	18	Office Action dated 02/09/2017 for U.S. Patent Application No. 15/076,565.	
	19	Office action dated 05/13/2019 for US Application No. 15/669,779.	
	20	Office action dated 05/20/2016 for US Application No. 14/855,301.	
	21	Office action dated 05/31/2016 for US Application No. 14/712,754.	
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	23	Office action dated 06/03/2016 for US Application No. 14/861,989.	
	24	Office action dated 06/12/2017 for US Application No. 15/492,659.	
	25	Office action dated 07/18/2017 for US Application No. 14/861,989	
	26	Office Action dated 07/30/2019 for U.S. Application No. 16/283,635.	
	27	Office action dated 08/06/2019 for US Application No. 14/855,301.	
	28	Office action dated 10/03/2013 for US Application No. 12/969,581.	
	29	Office Action dated 10/12/2016 for U.S. Patent Application No. 14/712,754.	
	30	Office action dated 10/20/2017 for US Application No. 14/425,189.	
	31	Office Action dated 11/21/2016 for U.S. Patent Application No. 14/855,301.	
	32	Office action dated 12/04/2015 for US Application No. 14/712,754.	
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	39	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Foundation Medicine Inc.	☐
	40	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Grunecker Patent-und Rechtsanwalte PartG mbB	☐
	41	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Personal Genome Diagnostics, Inc.	☐
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CERTIFICATION STATEMENT

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-16
Name/Print	Timothy A. Hott	Registration Number	67740

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Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-16
Name/Print	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Doc code: IDS
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(Not for submission under 37 CFR 1.99)

Application Number	16714579
Filing Date	2019-12-13
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.305

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	1	Guardant Health vs. FMI Invalidity Exhibit C-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	2	Guardant Health vs. FMI Invalidity Exhibit C-4, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	3	Guardant Health vs. FMI Invalidity Exhibit C-5, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	4	Guardant Health vs. FMI Invalidity Exhibit D-1, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	5	Guardant Health vs. FMI Invalidity Exhibit D-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	6	Guardant Health vs. FMI Invalidity Exhibit D-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	7	Guardant Health vs. FMI Invalidity Exhibit D-5, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	8	Guardant Health vs. FMI Second Amended Complaint dated March 6, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	9	Guardant Health vs. FMI Suppl Invalidity References dated February 15, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	
	10	Guardant Health vs. FMI Supplemental Invalidity Contentions dated March 29, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	
	11	Guardant Health vs. PGDx Amended Answer to Second Amended Complaint dated April 30, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	

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	12	Guardant Health vs. PGDx Invalidity Claim Chart A, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	13	Guardant Health vs. PGDx Invalidity Claim Chart B, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	14	Guardant Health vs. PGDx Invalidity Claim Chart C, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	15	Guardant Health vs. PGDx Invalidity Claim Chart D, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	16	Guardant Health vs. PGDx Invalidity Claim Chart E, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	17	Guardant Health vs. PGDx Invalidity Claim Chart F, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	18	Guardant Health vs. PGDx Invalidity Claim Chart G, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	19	Guardant Health vs. PGDx Invalidity Claim Chart H, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	20	Guardant Health vs. PGDx Invalidity Claim Chart I, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	21	Guardant Health vs. PGDx Invalidity Claim Chart J, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	22	Guardant Health vs. PGDx Invalidity Claim Chart K, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	

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	23	Guardant Health vs. PGDx Invalidity Claim Chart L, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	24	Guardant Health vs. PGDx Invalidity Contentions dated June 25, 2018 (C.A. No. 17-cv-1623-LPS-CJB)	
	25	Guardant Health vs. PGDx Second Amended Complaint dated March 23, 2018 (C.A. No. 17-cv-1623-LPS-CJB)	
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	50	Illumina "Preparing Samples for Paired-End Sequencing (2008)	

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	Attorney Docket Number	42534-708.305

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Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-16
Name/Print	Timothy A. Hott	Registration Number	67740

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	4	2013188471	WO	A2	2013-12-19	Faham et al.		
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	15	2015159293	WO	A2	2015-10-22	DOR et al.	Entire Document	
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	40	Guardant Health vs. FMI Invalidity Exhibit A-1, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	41	Guardant Health vs. FMI Invalidity Exhibit A-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
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That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

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Name/Print	Timothy A. Hott	Registration Number	67740

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☒ A certification statement is not submitted herewith.

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-01-16
Name/Print	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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