

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

GUARDANT HEALTH, INC.
Petitioner,

v.

UNIVERSITY OF WASHINGTON THROUGH ITS CENTER FOR
COMMERCIALIZATION
Patent Owner.

No. IPR2022-00817
Patent No. 10,760,127

**DECLARATION OF RAHUL SATIJA, D.PHIL IN SUPPORT OF PATENT
OWNER RESPONSE**

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I, Rahul Satija, hereby declare as follows.

I. INTRODUCTION

1. I have been retained on behalf of University of Washington through Its Center for Commercialization (“UW” or “Patent Owner”) for the above-captioned inter partes review proceeding to provide my expert opinions and expert knowledge. I understand that this proceeding involves U.S. Patent No. 10,760,127 (“the ’127 patent”) titled “Methods of Lowering the Error Rate of Massively Parallel DNA Sequencing Using Duplex Consensus Sequencing,” and that the ’127 Patent is currently assigned to UW.

2. I understand that in response to a Petition submitted by Guardant Health, Inc. (“Guardant” or “Petitioner”), an inter partes review of claims 1-30 of the ’127 patent was instituted by the Patent Trial and Appeal Board (“the Board”) on October 13, 2022.

3. In preparing this declaration, I have reviewed and am familiar with all the documents cited herein. I have reviewed and am familiar with the ’127 patent and its file history. I confirm that to the best of my knowledge the accompanying exhibits are true and accurate copies of what they purport to be, and that an expert in the field would reasonably rely on them to formulate opinions such as those set forth in this declaration.

4. I am being compensated at my customary rate of \$750 per hour for

my work on this case. My compensation is not dependent upon my opinions, my testimony, or the outcome of this case.

II. MY BACKGROUND AND QUALIFICATIONS

5. I am a Core Faculty Member at the New York Genome Center, and an Associate Professor with tenure at the Center for Genomics and Systems Biology at New York University. I also hold an appointment as an Associate Faculty Member at the Institute for Systems Genetics at NYU Langone Medical Center.

6. I was a postdoctoral fellow at the Broad Institute of Harvard and MIT from 2011-2014. My research focused on applying sequencing technologies to understand the behavior of immune cells, and I published articles describing my results in the journal Nature. I also conducted research on DNA sequencing technologies as a training fellow at the Medical Research Council in the United Kingdom from 2010-2011.

7. I received my D.Phil in Statistics at the University of Oxford in 2010. I attended Oxford University on a Rhodes Scholarship, and my graduate thesis focused on the statistical analysis of DNA sequencing data. I graduated summa cum laude from Duke University in 2006 with a Bachelor of Science in Biology, Mathematics, and Music.

8. The primary area of my research is the development and use of genomic technologies, such as technologies that involve DNA sequencing, as well

as the computational analysis of data related to these technologies.

9. I have a particular familiarity and expertise with genomic technologies for the analysis of nucleic acids present in miniscule samples, including DNA and RNA molecules that are present in single-cells. This includes the developments in molecular biology and microfluidics that enable the analysis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) from single cells, as well as technologies that evaluate the ability of thousands of different cells to produce specific protein molecules. My research is also focused on the development of statistical techniques involved in the analysis of this data.

10. I have over 15 years of experience in the field of molecular biology. I have taught undergraduate and graduate level courses at NYU in the area of sequencing technologies, computational analysis of genome sequencing data, and genetics.

11. My work has been published in more than 80 peer-reviewed scientific articles that have been cited more than 46,000 times. Since commencing my faculty appointment, I have presented more than 150 invited lectures, seminars, or workshops on my research. I have also received grant funding to continually support my research from multiple organizations including the National Institutes of Health, Chan Zuckerberg Initiative, Medical Research Council, and the Rhodes Trust.

12. I have received multiple awards and honors, including the “New Innovator Award” from the National Institutes of Health, and a selection as a Kavli Fellow from the National Academy of Sciences. In 2019, I was selected as a highly cited researcher by the Institute for Scientific Information. In 2020, I was selected to lead a Center of Excellence in Genomic Science by the National Human Genome Research Institute.

13. My Curriculum Vitae is attached to this declaration (EX2064), and contains additional details on my education, honors and awards, publications, professional activities, and qualifications to deliver an expert opinion in this matter.

III. SUMMARY OF OPINIONS

14. In preparing this declaration, I have reviewed the declaration of Dr. John Quackenbush, Ph.D. (EX1002). As detailed below, I disagree with Dr. Quackenbush’s arguments that the claims of the ’127 patent would have been obvious over the prior art. Dr. Quackenbush relies upon the combination of two references, Travers ’075 and Travers 2010, but fails to explain why or how a person of ordinary skill in the art (“POSA”) would have combined them to arrive at the ’127 patent claims. The teachings in the art would not have provided a POSA a reason to combine the rolling circle replication embodiment in Travers ’075 with the SMRT sequencing applications of Travers 2010. Moreover, these

references would not have provided a reasonable expectation of success.

15. In his analysis, Dr. Quackenbush fails to adequately address all the limitations of the challenged claims. First, Travers '075 and Travers 2010 do not teach obtaining first and second strand sequence reads, as recited in claims 1 and required by its dependent claims 2-21. Instead, these references teach the encoding of strand information on separate, but physically linked, regions on the same sequencing read. Dr. Quackenbush fails to address this issue in his declaration.

16. Second, Travers '075 and Travers 2010 do not teach the grouping of multiple sequence reads into families based on barcode sequences and fragment-specific information, as recited in claim 22. While Dr. Quackenbush argues that Travers '075 teaches the use of *sample* barcodes, enabling researchers to simultaneously obtain sequencing data from multiple *samples*, these barcodes are unrelated to the limitations in claim 22 (and its dependent claims 23-30). Moreover, these barcodes must be identical for all template molecules within a sample, and therefore are not fragment-specific.

17. Third, Travers '075 and Travers 2010 do not teach the use of the asymmetric nature of DNA-adapter sequences to distinguish the first and second strands of an original adapter-DNA molecule as recited in claim 19. Travers '075 and Travers 2010 teach the use of the DNA-adapter molecules to mark the location where one strand segment ends and another strand segment begins. Both references

disclose using these adapter sequences as landmarks, or “registration sequences”, and never teach the use of any asymmetric nature of the sequence in order to distinguish strand information.

18. In addition, Dr. Quackenbush neglected to address or take into account multiple problems associated with using a rolling circle template replication technique disclosed in Travers '075 to produce linear template concatemer molecules, and then sequence those concatemers using the SMRT sequencing applications of Travers 2010. Those problems would have been apparent to a POSA.

19. As discussed below, the claimed methods of the '127 patent require a separate template amplification step after adapter ligation and before sequencing. However, a POSA would have understood that the SMRT sequencing workflow disclosed in Travers '075 and Travers 2010 does not utilize a DNA template amplification step, and that the absence of this step was a crucial advantage. As discussed below, there are several critical problems associated with performing rolling circle replication to produce linear concatemers that are then sequenced using SMRT sequencing. Addressing these problems would have required undue experimentation and indeed, it is unlikely that a POSA could have even made such an embodiment work.

20. Dr. Quackenbush's opinions are flawed because a POSA would not

have had a reason to include an additional rolling circle replication step in the well-established SMRT sequencing methods. A POSA would have understood that the traditional SMRT sequencing applications in Travers 2010 and Travers '075 utilize circle sequencing, which involves sequencing the same circular template molecule over and over again. As of March 20, 2012, it was known that SMRT sequencing was plagued by an inherently high per-base error rate, and therefore utilized circle sequencing to provide the necessary sequencing redundancy to generate more reliable sequencing outputs. A POSA would have understood that modifying the SMRT workflow to perform an amplification step after ligation would not only be unnecessary but introduce significant technical problems, which would have been appreciated by a POSA.

21. Dr. Quackenbush also failed to account for the fact that Travers '075's rolling circle replication embodiment would produce a set of linear molecules that are incompatible with the SMRT sequencing workflow, and would require significant undue experimentation to generate any usable sequencing data for multiple reasons, as detailed below.

22. Dr. Quackenbush failed to consider that Travers '075's rolling circle replication embodiment would not have been expected to produce any useable sequencing data at all. This is because the long, linear concatemer generated from Travers '075's embodiment would have contained repeating copies of the

sequencing primer/polymerase binding sites. A POSA would have understood that this concatemer would bind multiple polymerase molecules and render this multi-complex unsequenceable on the ZMW arrays for SMRT sequencing. Thus, this reason alone would have dissuaded a POSA from incorporating a rolling circle replication step in the SMRT sequencing applications of Travers 2010.

23. A POSA would know that rolling circle replication produces a wide range of products, ranging in size from only a few hundred nucleotides to large concatemers several thousands nucleotides long. However, it was well-known as of March 20, 2012 that SMRT sequencing suffered from a bias, driven by differences in diffusion between small and large DNA molecules, that would have allowed smaller template molecules to significantly outcompete larger template molecules for occupancy and sequencing in these ZMW wells.

24. This physical reality resulted in a limitation in the SMRT sequencing workflow: sequencing a mixture of small and large fragments that both would not result in usable sequencing data. First, a POSA would have recognized that even though the smaller template molecules generated from rolling circle replication could diffuse into the ZMW wells, they would contain few copies of the target DNA and thus would not provide the required sequencing redundancy for SMRT sequencing's error correction. Dr. Quackenbush fails to address this issue in his declaration, and admitted at his deposition that he did not consider DNA diffusion

at all when preparing his declaration. Second, a POSA would have understood that the larger concatemer template molecules would be outcompeted by the smaller molecules for diffusion into the ZMW wells for SMRT sequencing.

25. In addition to the slower diffusion of the concatemer molecules generated from Travers '075's rolling circle replication embodiment, the narrow diameter of the ZMWs would have precluded the large concatemer molecules from physically fitting into the wells. Indeed, I have shown through in silico modeling that the repeating complementary stretches of DNA in these large concatemers would hybridize to form complex secondary structures that would have difficulties physically fitting into the narrow ZMW wells for SMRT sequencing. Dr. Quackenbush also fails to address this issue in his declaration.

26. Moreover, regarding claim 13, a POSA would not have been motivated to combine the Travers Publications and McCloskey. Dr. Quackenbush argues that McCloskey's disclosure of degenerate barcode sequences renders claim 13 obvious. However, McCloskey deals exclusively with PCR, and relates to amplification methods that generate multiple, separate, and unlinked molecules that are copies of the same template sequence. By contrast, rolling circle amplification creates a linear product where all amplification-derived copies are physically connected in a single concatemer molecule. A POSA would have no reason to utilize the teachings of McCloskey when performing rolling circle

amplification.

27. In sum, a POSA would not have had any reason to combine Travers '075's rolling circle replication embodiment with the SMRT sequencing applications of Travers 2010. Moreover, even in the absence of this motivation, a POSA attempting to combine these embodiments would have run into serious technical challenges and functional incompatibilities, and would not have had a reasonable expectation of success without excessive and undue experimentation.

IV. LIST OF DOCUMENTS CONSIDERED

In formulating my opinions, I have reviewed all the references and documents cited herein, including those listed below.

Exhibit No.	Description
1001	U.S. Patent No. 10,760,127 to Salk et al.
1002	Declaration of Dr. John Quackenbush
1003	<i>Curriculum Vitae</i> of John Quackenbush, Ph.D.
1004	File History for Application No. 16/503,382
1006	Kinde et al., "Detection and quantification of rare mutations with massively parallel sequencing," <i>PNAS</i> 108(23) (2011)

Exhibit No.	Description
1009	U.S. Patent Publication No. 2012/0071331 to Casbon et al.
1010	Shiroguchi et al., “Digital RNA sequencing minimizes sequence dependent bias and amplification noise with optimized singlemolecule barcodes,” <i>PNAS</i> 109(4) (2012)
1012	Illumina, TruSeq RNA and DNA Sample Preparation Kits, Data Sheet: Illumina Sequencing (2010)
1013	Bentley et al., “Accurate whole human genome sequencing using reversible terminator chemistry,” <i>Nature</i> 456 (2008)
1014	Evans et al., “NEB Expressions” New England BioLabs (2007)
1015	NEB Expressions, New England BioLabs, https://web.archive.org/web/20080321144426/http://www.neb.com/nebecomm/tech_reference/neb_transcripts.asp (Feb. 23, 2022)
1016	Affidavit of Nathaniel E. Frank-White
1017	Nisha et al., “Antioxidants and Their Protective Action Against DNA Damage,” <i>International Journal of Pharmacology and Pharmaceutical Sciences</i> 3 (2011)
1018	U.S. Patent Publication No. 2009/0298075 to Travers et al.
1020	U.S. Patent No. 7,476,503 to Turner et al.

Exhibit No.	Description
1021	Travers et al., “A flexible and efficient template format for circular consensus sequencing and SNP detection,” <i>Nucleic Acids Research</i> 38(15) (2010)
1022	International Publication No. WO 2012/142213 to Vogelstein et al.
1023	U.S. Patent Publication No. 2007/0020640 to McCloskey et al.
1024	File History for Application No. 16/118,306
1025	File History for Application No. 17/392,185
1026	McCarthy, “Third Generation DNA Sequencing: Pacific Biosciences’ Single Molecule Real Time Technology,” <i>Chemistry & Biology</i> 17 (2010)
1027	Song et al., “Sensitive and specific single-molecule sequencing of 5-hydroxymethylcytosine,” <i>Nature Methods</i> 9(1) (2012)
1028	Hodges et al., “Genome-wide <i>in situ</i> exon capture for selective resequencing,” <i>Nature Genetics</i> 39(12) (2007)
1030	Clark et al., “Direct Detection and Sequencing of Damaged DNA Bases,” <i>Genome Integrity</i> 2 (2011)
1031	Casbon et al., “A method for counting PCR template molecules with application to next-generation sequencing,” <i>Nucleic Acids Research</i> 39(12)(2011)

Exhibit No.	Description
2001	Eid, J., et al., “Real-Time DNA Sequencing from Single Polymerase Molecules,” <i>Science</i> 323:133-138 (2009)
2002	Niedringhaus, T.P., et al., “Landscape of Next-Generation Sequencing Technologies,” <i>Anal. Chem.</i> 83:4327-4341 (2011)
2003	Thompson, J.F. and Milos, P.M., “The Properties and Applications of Single-Molecule DNA Sequencing,” <i>Genome Biology</i> 12:217 (2011)
2004	Stranneheim, H. and Lundeberg, J., “Stepping Stones In DNA Sequencing,” <i>Biotechnol</i> 7:106-1073 (2012)
2005	Quail, M.A., et al., “A Tale of Three Next Generation Sequencing Platforms: Comparison of Ion Torrent, Pacific Biosciences and Illumina Miseq Sequencers,” <i>BMC Genomics</i> 13:341 (2012)
2006	Egan, A.N., et al., “Applications of Next-Generation Sequencing In Plant Biology,” <i>American Journal of Botany</i> 99(2):175-185 (2012)
2007	Kirsch, S. and Klein, C.A., “Sequence Error Storms and the Landscape of Mutations in Cancer,” <i>Proc. Natl. Acad. Sci.</i> 109(36):14289-14290 (2012)
2008	Turner, S., et al., “Apparatus and Method for Performing Nucleic Acid Analysis,” U.S. Patent No. 7,479,503 B2 (filed Sept. 16, 2005; issued Jan 13, 2009)
2016	Decision Denying Institution, <i>Guardant Health, Inc. v. University of Washington</i> , Case No. IPR2022-00816 (P.T.A.B.), Paper No. 16, filed Oct. 13, 2022
2017	Johne, R., et al., “Rolling-Circle Amplification Of Viral DNA Genomes Using Phi29 Polymerase,” <i>Trends in Microbiology</i> , 17(5):205-11 (2009)

Exhibit No.	Description
2019	Guardant Health, Inc. Response to Notice of Opposition, European Patent No. 2,893,040, filed May 29, 2020
2020	Prosecution History of U.S. Patent No. 10,760,127
2021	“Single Molecule Real Time (SMRT™) DNA Sequencing,” Technology Backgrounder, <i>Pacific Biosciences</i> (2009)
2022	Fichot, E. B. and Norman, R. S, “Microbial Phylogenetic Profiling With The Pacific Biosciences Sequencing Platform,” <i>Microbiome</i> 1(10):1-5 (2013)
2023	Schadt, E. E., et al., “A Window Into Third-Generation Sequencing,” <i>Pacific Human Molecular Genetics</i> , 19(2):R277-R240 (2010)
2024	Liu, L., et al., “A Brief Utilization Report On The Illumina Hiseq 2000 Sequencer,” <i>Mycology</i> , 2(3):169-91 (2011)
2025	Koren, S., et al., “Hybrid Error Correction And <i>De Novo</i> Assembly Of Single-Molecule Sequencing Reads,” <i>Nature Biotechnology</i> (2012)
2026	Eid, J, et al., “Supporting Online Material for: Real-Time DNA Sequencing from Single Polymerase Molecules,” <i>Science</i> (2008)
2027	Gupta, P. K., “Single-Molecule DNA Sequencing Technologies For Future Genomics Research,” <i>Trends in Biotechnology</i> , 26(11):602-11 (2008)
2028	Zhang, X., et al., “Improving Genome Assemblies By Sequencing PCR Products With Pacbio,” <i>BioTechniques</i> , 53(1):61-2 (2012)

Exhibit No.	Description
2029	Levene, M. J., et al., “Zero-Mode Waveguides For Single-Molecule Analysis At High Concentrations,” <i>Science</i> , 299:682-86 (2003)
2030	Korlach, J., et al., “Selective Aluminum Passivation For Targeted Immobilization Of Single DNA Polymerase Molecules In Zero-Mode Waveguide Nanostructures,” <i>PNAS</i> , 105(4):1176-81 (2008)
2031	McCarthy, A., “Third Generation DNA Sequencing: Pacific Biosciences’ Single Molecule Real Time Technology,” <i>Chemistry & Biology</i> , 17:675-76 (2010)
2032	Shendure, J. A., et al., “Overview of DNA Sequencing Strategies,” <i>Current Protocols in Molecular Biology</i> , Supp. 96:7.1.1-7.1.23 (2011)
2033	Mardis, E. R., “Next-Generation Sequencing Platforms,” <i>Annual Review of Analytical Chemistry</i> , 6:287-303 (2013)
2034	Glenn, T. C., “Field Guide To Next-Generation DNA Sequencers,” <i>Molecular Ecology Resources</i> , 11:759-69 (2011)
2035	Ozsolak, F., “Third Generation Sequencing Techniques and Applications to Drug Discovery,” <i>Expert Opinion on Drug Discovery</i> , 7(3):231-43 (2012)
2036	Demidov, V. V., “Rolling-Circle Amplification In DNA Diagnostics: The Power Of Simplicity,” <i>Expert Review of Molecular Diagnostics</i> , 2(6):542-48 (2002)
2037	Liu, D., et al., “Rolling Circle DNA Synthesis: Small Circular Oligonucleotides as Efficient Templates for DNA Polymerases,” <i>Journal of American Chemical Society</i> , 118:1587-94 (1996)
2038	Lizardi, P. M., et al., “Mutation Detection And Single-Molecule Counting Using Isothermal Rolling-Circle Amplification,” <i>Nature Genetics</i> , 19:225-32

Exhibit No.	Description
2039	Smith, D. E., et al., “Dynamical Scaling of DNA Diffusion Coefficients,” <i>Macromolecules</i> , 29:1372-73 (1996)
2040	Robertson, R. M., “Diffusion Of Isolated DNA Molecules: Dependence On Length And Topology,” <i>Proceedings of the National Academy of Sciences</i> , 103(19):7310-14 (2006)
2041	Grosberg, A. Y. and Rabin, Y., “DNA Capture Into A Nanopore: Interplay Of Diffusion And Electrohydrodynamics,” <i>The Journal of Chemical Physics</i> , 133: 165102-1-15 (2010)
2042	Coupland, P., et al., “Direct Sequencing Of Small Genomes On The Pacific Biosciences RS Without Library Preparation,” <i>BioTechniques</i> , 53(6): 365-72 (2012)
2045	Deposition Transcript of John Quackenbush, Ph.D., <i>Guardant Health, Inc. v. University of Washington</i> , Case No. IPR2022-00817 (P.T.A.B), Dec. 19, 2022
2046	Loomis, E. W., et al., “Sequencing The Unsequenceable: Expanded CGG-Repeat Alleles Of The Fragile X Gene,” <i>Genome Research</i> , 23:121-28 (2014)
2047	Drmanac, R., et al., “Human Genome Sequencing Using Unchained Base Reads on Self-Assembling DNA Nanoarrays,” <i>Science</i> , 327: 78-81 (2010)
2048	Hui, P., “Next Generation Sequencing: Chemistry, Technology and Applications,” <i>Topics in Current Chemistry</i> (2012)
2049	U.S. Provisional Application No. 61/072,160 to Patel et al., filed Mar. 28, 2008

Exhibit No.	Description
2050	Schmitt, M. W., et al., “Detection Of Ultra-Rare Mutations By Next-Generation Sequencing,” <i>Proceedings of The National Academy of Sciences</i> , 109(36):14508-13 (2012)
2051	Kelvey, J., “Liquid Biopsies Generate Ample Insights from Sparse Clues,” Genetic Engineering & Biotechnology News (2020)
2052	LeMieux, J., “Sequencing’s Stars Urge Exploration of New Dimensions” Genetic Engineering & Biotechnology News, 40(4) (2020)
2053	“TwinStrand Biosciences to Showcase Recent Applications of its Duplex Sequencing Technology at the 2022 AGBT Annual Meeting,” Businesswire (2022)
2054	Ribeiro, F. J., et al., “Finished Bacterial Genomes From Shotgun Sequence Data,” <i>Genome Research</i> , 22:2270-77 (2012)
2055	“Quality Scores for Next-Generation Sequencing,” Illumina (2011)
2056	“12A: Nomenclature,” Encyclopedia of Separation Science, Academic Press, 4712-53 (2000) (excerpt)
2057	Larkin, J., et al., “Length-Independent DNA Packing into Nanopore Zero-Mode Waveguides for Low-Input DNA Sequencing,” <i>Nature Nanotechnology</i> , 12(12) 1169-75 (2017)
2058	Kalendar, R., et al., “Java Web Tools For PCR, In Silico PCR, And Oligonucleotide Assembly And Analysis,” <i>Genomics</i> , 98:137-44 (2011)

Exhibit No.	Description
2059	Kalendar, R., et al., “Java Web Tools For PCR, In Silico PCR, And Oligonucleotide Assembly And Analysis,” <i>Genes, Genomes and Genomics</i> , 3(1):1-14 (2009)
2060	Vincze, T., et al., “NEBcutter: A Program To Cleave DNA With Restriction Enzymes,” <i>Nucleic Acids Research</i> , 31(13):3688-91 (2003)
2061	Reuter, J. S., “RNAstructure: Software For RNA Secondary Structure Prediction And Analysis,” <i>BMC BioInformatics</i> , 11(129):1-9 (2010)
2062	Kennedy, S. R., et al., “Detecting Ultralow-frequency Mutations by Duplex Sequencing,” <i>Nature Protocols</i> , 9(11):2586-2606 (2014)
2063	Ng, D. W. and Wong, C. K. B, “Self-Recognition Of DNA From Life Processes To DNA Computation,” <i>Biophysical Reviews and Letters</i> , 2(2):123-37 (2007)
2064	<i>Curriculum Vitae</i> of Rahul Satija, D.Phil
2065	“TwinStrand’s Duplex Sequencing Goes Deep,” GenEdge, Genetic Engineering & Biotechnology News (2020)
2066	Semsarian, C., et al., “Molecular Medicine in the 21 st Century,” <i>Internal Medicine Journal</i> , 31:53-59 (2001)
2067	Forthofer, R. N., “Biostatistics: A Guide to Design, Analysis, and Discovery,” Chapter 5: Probability Distributions, Elsevier Academic Press, 103-33 (2007) (excerpt)

Exhibit No.	Description
2068	Esteban, J. A., et al., “Fidelity of Φ 29 DNA Polymerase,” The Journal of Biological Chemistry, 268(4):2719-26 (1993)
2069	DNA Secondary Structures for 6x Product
2070	DNA Secondary Structures for 10x Product
2071	DNA Secondary Structures for 20x Product

V. PERSON OF ORDINARY SKILL IN THE ART

28. I understand that a person of ordinary skill in the art (“POSA”) is a hypothetical person who is presumed to be aware of all pertinent art, thinks along conventional wisdom in the art and is a person of ordinary creativity.

29. A POSA relevant to the ’127 patent would have had knowledge of the scientific literature concerning methods of DNA manipulation and analysis, including DNA sample preparation for sequencing, amplification, methods of DNA sequencing (including NGS and related sequencing methods), and bioinformatics methods for raw data analysis.

30. A POSA would have had an advanced degree, such as a Ph.D., in molecular biology, genomics, bioinformatics, or a related field, and have at least

about two years of experience in the use and development of sequencing technologies. A POSA could also be a person with a lower level of formal education and more years of experience in one of these fields. Thus, I apply Dr. Quackenbush's definition of a POSA. EX1002, ¶¶46-47.

VI. STATE OF THE ART

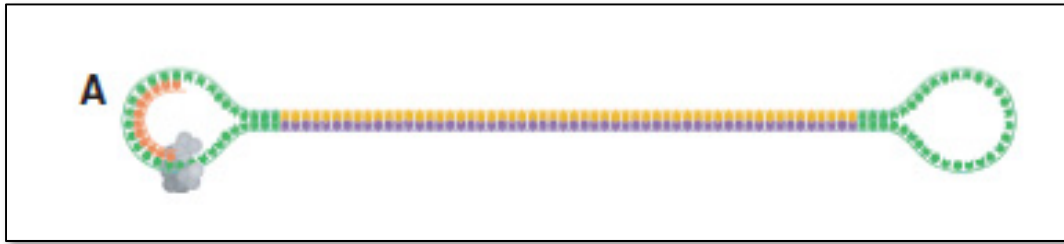
A. Travers '075 and Travers 2010 teach the use of DNA sequencing technology called SMRT sequencing.

31. Dr. Quackenbush relies on disclosures of sample preparation and sequencing methods developed by Pacific Biosciences of California, Inc. ("PacBio") taught in Travers '075 (EX1018) and the Travers Paper ("Travers 2010") (EX1021), collectively known as the Travers Publications. EX1002, ¶¶40-43.

32. Travers '075 and Travers 2010 teach using PacBio's DNA sequencing template design called "SMRTbell" for use in "single-molecule real-time (SMRT™)" sequencing methods. EX1021, 1; EX1018, ¶[0141]. Both Travers '075 and Travers 2010 teach ligating hairpin adapters to each end of a template DNA molecule to generate a circular SMRTbell template that is then ready for single-molecule sequencing in a series of nanowells called zero mode waveguides (ZMWs). EX1021, 4, Fig. 2; EX1018, ¶¶[0105], [0141]-[0148], Fig. 7.

33. As illustrated below in Figure 1, Travers 2010's SMRTbell template

“consists of a double-stranded region (the insert) flanked by two hairpin loops. The hairpin loops present a single-stranded region to which a sequencing primer can bind (orange).” EX1021, 3.



EX1021, 3, Fig. 1 (excerpted). As shown in Travers 2010’s figure above, the SMRTbell template is symmetrical between the top and bottom strands. EX1021, 3, Fig. 1. Indeed, Travers 2010 teaches that “[i]n deciding on a format for SMRT™ sequencing, a number of factors were considered, including ... *uniformity of structure....*” Travers 2010 discloses ligating the hairpin adapters to each end of the symmetrical DNA molecule to generate the circular SMRTbell template shown in Figure 1. EX1021, 4, Fig. 2. Travers 2010 then teaches that these circular molecules are “then applied to SMRT™ sequencing.” EX1021, 4, Fig. 2.

34. Travers 2010 explains that SMRT sequencing is “a method for generating sequence data that harnesses the intrinsic speed, fidelity and processivity of polymerase molecules” and cites the Eid 2009 reference for support. EX1021, 1; EX2001. Travers 2010 also states that “SMRT™ sequencing [is] described in [Eid 2009].” EX1021, 4. Eid 2009, which was coauthored by

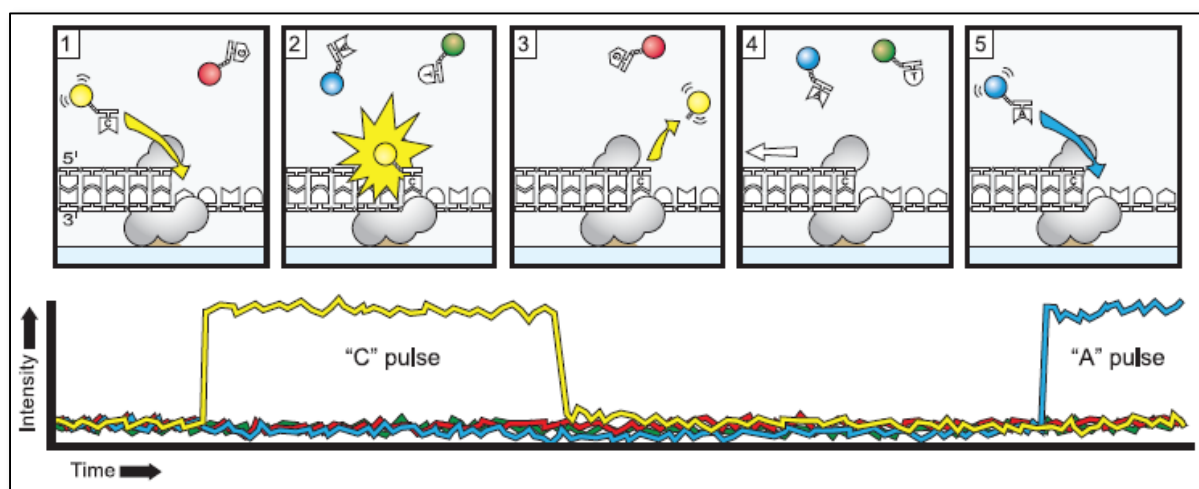
Travers, is PacBio's seminal publication disclosing the SMRT sequencing method. EX2001, 133.

35. Specifically, Eid 2009 teaches that SMRT™ sequencing involves obtaining “single-molecule, real-time sequencing data” from a DNA polymerase performing uninterrupted template-directed synthesis using dNTPs by detecting the “temporal order of their enzymatic incorporation into a growing DNA strand with zero-mode waveguide [ZMW] nanostructure arrays.” EX2001, Abstract. Eid 2009 goes on to state that “[c]onjugation of fluorophores to the terminal phosphate moiety of the dNTPs allows continuous observation of DNA synthesis over thousands of bases without steric hindrance.” EX2001, Abstract.

36. In other words, according to Eid 2009, Travers 2010's SMRT sequencing involves monitoring and recording in real-time the incorporation of fluorescently labeled nucleotides by a DNA polymerase as it moves along the template molecule, with each nucleotide incorporation event emitting a different color fluorophore that is captured by a camera. EX2001, 133-134; EX1021, 4. Travers 2010 discloses, as does Eid 2009, that “[n]ucleotide analogs, each containing a fluorophore linked to its terminal phosphate, are incorporated into a nascent chain” and that “fluorophores are cleaved during incorporation, leaving a native product and a free fluorophore.” EX1021, 4. Travers 2010 teaches detecting the fluorescence signals of each incorporation event with a camera: “[t]he

fluorescence signal for each of the analogs is observed by a CCD camera.”

EX1021, 4-5. Eid 2009 depicts the SMRT sequencing process in Figure 1, shown below:



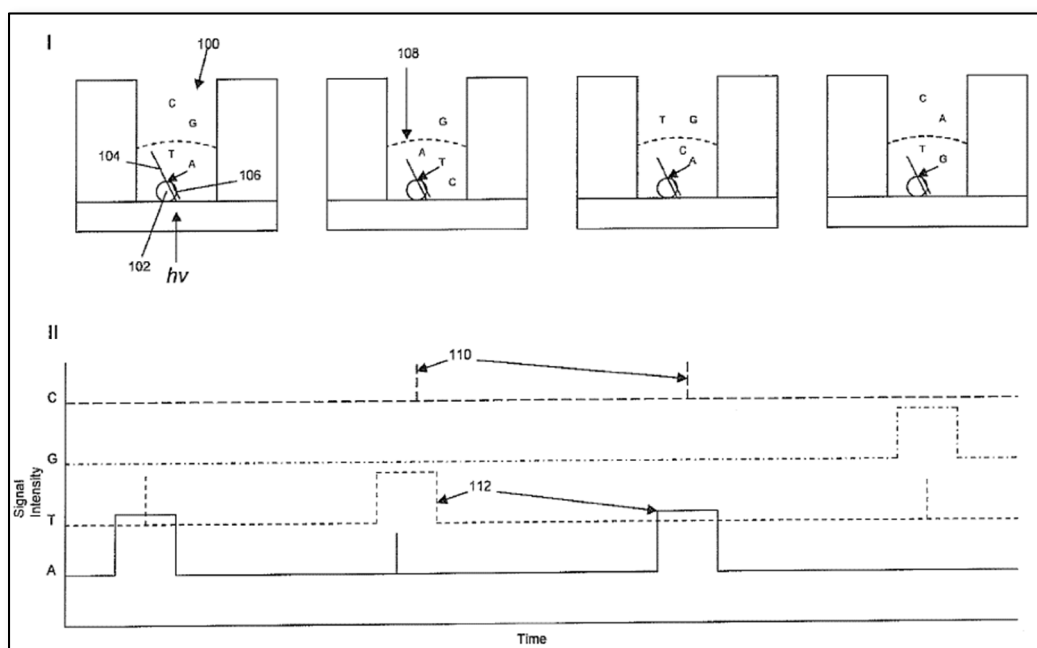
EX2001, Fig. 1 (excerpted); *see also*, EX1021, 4.

37. As can be seen in the figure above in Eid 2009, incorporation of the fluorescently labeled “C” nucleotide by DNA polymerase results in a transient yellow fluorescence emission (step 2, “C” pulse), followed by incorporation of a fluorescently labeled “A” nucleotide which will result in a transient blue fluorescence emission (steps 5, “A” pulse). EX2001, Fig. 1; EX1021, 4. These color-coded emissions, each representing a different nucleotide A for adenine, T for thymine, C for cytosine, or G for guanine, are captured by the camera and assembled into a nucleotide sequence in real time. EX1021, 4-5.

38. Thus, a POSA would have understood from Travers 2010 and Eid

2009 that strand extension of the SMRTbell template molecule (performed by a DNA polymerase enzyme) *is* the sequencing reaction (fluorescent flashes captured by a camera) in Travers 2010's SMRT™ sequencing workflow. EX1021, 4-5, EX2001, Fig 1.

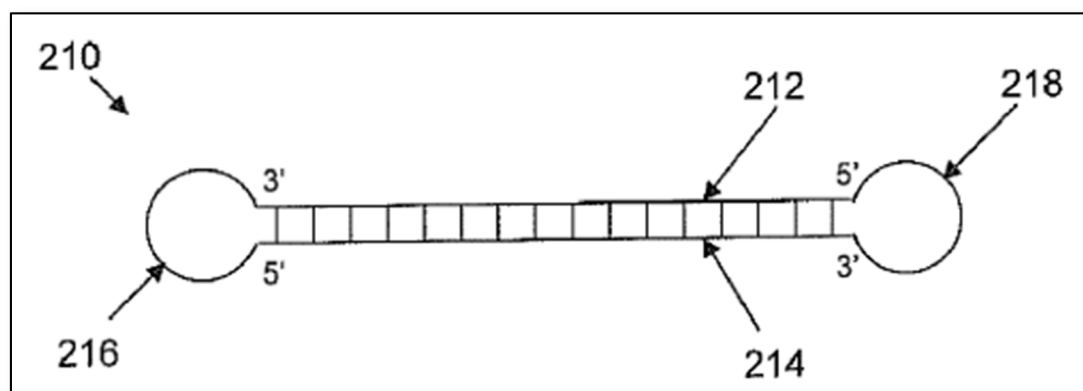
39. Dr. Quackenbush admits that Travers '075 discloses the same SMRT sequencing methods and SMRTbell template molecules disclosed in Travers 2010. EX1002, ¶42. Travers '075 discloses that an “exemplary sequencing process based upon such Single Molecule Real Time (SMRT™) processes is schematically illustrated in FIG. 1,” and provides a figure similar to Eid 2009's Figure 1 shown above:



EX1018, ¶¶[0042]-[0043], Fig. 1A. Travers '075 teaches that in Panel I of Fig. 1, a

“nucleic acid synthesis complex comprising a polymerase enzyme 102, a template sequence 104, and a primer sequence 106 complementary to a portion of the template sequence 104 is provided immobilized within a confined illumination volume (indicated by the dashed line 108.” EX1018, ¶[0043], Fig. 1.

40. Travers '075 goes on to disclose that “[t]he reaction mixture ... contains the four different nucleotides (A, G, T, and C) each labeled with a spectrally distinguishable fluorescent label attached through its terminal phosphate group.” EX1021, ¶[0044]. Travers '075 then teaches that by identifying the “different spectral characteristics, one can detect, in real time, the identity of each incorporated base as it is being incorporated.” EX1021, ¶[0045]. As shown below, Figure 2 of Travers '075 also discloses the same circular SMRTbell template format shown in Figure 1 of Travers 2010:



EX1018, Fig. 2B, ¶[0050].

B. The SMRT™ sequencing methods of Travers '075 and Travers 2010 do not use a SMRTbell template amplification step before sequencing.

41. Unlike the claimed methods in the '127 patent, which recite attaching adapters to DNA molecules, then amplifying the adapter-DNA molecules, and then sequencing the amplified adapter-DNA molecules in separate, discrete steps, the circular SMRTbell template molecules taught in Travers 2010 are subjected *directly* to sequencing, with no SMRTbell template amplification step. *See* EX1021, 4. The art referred to this process as “sequencing by synthesis.” EX2003, 5; EX2004, 8; EX2021, 1, 6; EX2022, 1; EX1018, ¶[0083]. A POSA would have known that SMRT sequencing’s version of sequencing by synthesis is not a template amplification step. EX2003, 5; EX2004, 8; EX2021, 1, 6; EX2022, 1; EX1018, ¶[0083]. Indeed, Travers 2010 expressly discloses that “this protocol for template preparation requires a minimal number of steps and *does not depend on amplification*.” EX1021, 5 (emphasis added)¹.

42. Travers '075 also teaches that the SMRT sequencing methods do not amplify the SMRTbell templates before sequencing. In fact, Travers '075 teaches that “the constructs of the invention are described primarily, and preferably, for *use directly as templates* for, e.g., sequencing applications....” EX1018, ¶[0122]

¹ Emphasis is added throughout this declaration, unless otherwise noted.

(emphasis added).

43. Travers '075 also praises the use of SMRTbell templates and discloses that “[t]he templates of the invention provide numerous advantages over simple linear template sequences,” explaining that its circular SMRTbell templates offer “the expectation that the entire template will be sequenced or at least sequenceable.” EX1018, ¶¶[0053], [0063]. However, using linear templates creates a “risk of missing upstream portions of the template for sequencing.” EX1018, ¶¶[0053], [0063].

44. This understanding is also confirmed in Travers '075's working examples, none of which discloses amplifying SMRTbell template molecules before sequencing. In Example 1, Travers '075 discloses generating a “final template construct” and then directly performing “[t]he SMRT™ sequencing reaction” without an intervening template amplification step. EX1018, ¶¶[0139]-[0140]. Similarly, Example 2 in Travers '075 discloses that after template construction, “[t]he resulting template was subjected to SMRT™ sequencing” without a template amplification step. EX1018, ¶[0142]. Example 3 is no different. Travers '075 discloses that after template construction, “[t]he templates were annealed to an equivalent amount of primer, and then subjected to sequencing” without a template amplification step. EX1018, ¶[0145]. Example 4 also discloses that after template construction, “[t]he template was then sequenced using a

SMRT™ sequencing” with no template amplification step performed before sequencing. EX1018, ¶[0151].

45. None of the claims in Travers ’075 teach including a template amplification step after adapter ligation and before performing the SMRT™ sequencing method. The methods claimed in Travers ’075 recite “contacting the template nucleic acid segment with a primer sequence” and then “monitoring a template directed, polymerase mediated nucleic acid sequencing process to determine a sequence of nucleotides.” EX1018, claim 12.

46. It is not surprising that none of the claims or examples in Travers ’075 teach the use of a template amplification step after adapter ligation and prior to sequencing. As discussed below, it was well established in the art that not only did the SMRT sequencing method not rely on such an amplification step, but this lack of reliance represented a distinct and important advantage of SMRT sequencing. While such pre-amplification steps were requirements of alternative sequencing workflows, they inevitably introduce errors and biases that may reduce the quality and reliability of the downstream sequencing data.

47. Dr. Quackenbush explains that Travers 2010 is authored “by scientists employed by PacBio (including inventors of Travers ’075) and describes applications of the sequencing method described in Travers ’075.” EX1002, ¶¶41-42. As discussed above, the PacBio SMRT sequencing workflow of Travers ’075

and Travers 2010 is different from the claimed methods in the '127 patent, which require a separate amplification step prior to the sequencing step. And as further detailed herein, a POSA would not have been motivated to include a separate rolling circle replication step in the PacBio SMRT sequencing workflow after adapter ligation and before sequencing as required by all claims of the '127 patent.

VII. THE '127 PATENT CLAIMS

48. The '127 patent is directed to DNA-sequencing methods that employ error-correction technology. EX1001, Abstract. The claims generally cover duplex sequencing methods that involve tagging sample DNA strands with short nucleic-acid identifiers to track their origin and identity through various processing steps. EX1001, Abstract. Duplex sequencing involves tagging both strands of a sample of double-stranded DNA fragments with asymmetric adapters to keep track of which strand is which (i.e. the “first” and “second” strands; sometimes called “top” and “bottom” strands). EX1001, Abstract, Fig. 1. By tracking the DNA strand origin in this asymmetric fashion throughout sequencing and further processing, duplex sequencing methods can dramatically improve sequencing accuracy by comparing strands against their complementary strand. Using this approach, duplex sequencing can distinguish actual mutations (which will be detected on sequencing reads representing both strands) from sequencing errors and other technical artifacts (which will be detected on sequencing reads representing only one strand).

EX1001, Abstract, Fig. 3.

49. I understand that the '127 patent has 30 claims in total. I also understand that there are two independent claims, claims 1 and 22, from which all other claims depend.

VIII. THE MEANING OF CLAIM TERMS (CLAIM CONSTRUCTION)

50. I understand that terms of the claims generally are given their ordinary and customary meaning in light of the specification and prosecution history—that is, the meaning that the term would have to a POSA in question at the time of the invention. This is the standard I applied here.

A. “Amplifying” adapter-DNA molecules to produce “strand copies”

51. A POSA would understand that independent claims 1 and 22 recite separate steps of (a) attaching adapters to double-stranded DNA fragments; (b) amplifying the adapter-DNA fragments to produce “strand copies”; (c) sequencing the “strand copies” or amplified adapter-DNA fragments; and then (d) assessing sequencing information through a series of steps involving grouping sequence reads, comparing sequence read information between the strands, and analyzing the sequences to identify a sequence variation. EX1001, claims 1 and 22.

52. A POSA would understand from reading these claims that the amplifying step (b) in claims 1 and 22 is a separate step that occurs *after* the adapter attachment step (a) and *before* the sequencing step (c). Claims 1 and 22 are

shown below.

Claim 1	Claim 22
1. A method of sequencing DNA comprising;	22. A method of sequencing DNA comprising:
a) <i>attaching adapters</i> to double-stranded DNA fragments to generate a plurality of partially-complementary, asymmetrical <i>double-stranded adapter-DNA molecules</i> , wherein the adapters comprise barcodes selected from a plurality of distinct barcode sequences;	a) <i>attaching</i> partially single-stranded <i>adapters</i> comprising barcodes selected from a plurality of distinct barcode sequences to double-stranded DNA fragments obtained from a bodily sample, wherein attachment of the adapters to double-stranded DNA fragments generates a library of tagged double-stranded <i>adapter-DNA molecules</i> ;
b) <i>amplifying</i> original strands of at least a portion of the double-stranded <i>adapter-DNA molecules</i> to <i>produce first and second strand copies</i> ;	b) <i>amplifying</i> strands from a plurality of the double-stranded <i>adapter-DNA molecules</i> in the library to <i>produce strand copies</i> ;
c) <i>sequencing</i> a plurality of <i>first and second strand copies</i> to obtain first and second strand sequence reads for at least a portion of the adapter-DNA molecules; and	c) <i>sequencing</i> a plurality of <i>the strand copies</i> to obtain strand sequence reads comprising one or more barcode sequences and DNA fragment-specific information; and

Claim 1	Claim 22
<p>d) for at least some of the adapter-DNA molecules comprising barcodes</p> <p>confirming the presence of at least one sequence read derived from each of the original first and second strands of the adapter-DNA molecules;</p> <p>comparing at least one of the confirmed first and second strand sequence reads to a reference sequence; and</p> <p>analyzing one or more correspondences between at least one of the confirmed first and second strand sequence reads and the reference sequence to identify a sequence variation.</p>	<p>d) for at least some of the double-stranded adapter-DNA molecules in the library</p> <p>grouping the strand sequence reads into families based on i) the barcode sequence, and ii) DNA fragment-specific information;</p> <p>collapsing a plurality of strand sequence reads within the families to provide a consensus sequence for each of at least some of the double-stranded DNA molecules in the library;</p> <p>comparing the consensus sequence to a reference sequence; and</p> <p>analyzing one or more correspondences between the consensus sequence and the reference sequence to identify a sequence variation.</p>

53. As shown above, claims 1 and 22 each require the following: (a) attaching adapters to double-stranded DNA fragments to generate “adapter-DNA molecules”; (b) amplifying the adapter-DNA molecules to produce “strand copies”; (c) sequencing the “strand copies” that were produced in the amplification step (b). EX1001, claims 1 and 22. A POSA reviewing the claims would have understood that the amplification step is a separate step occurring after adapter attachment and before sequencing.

54. The plain language of the claims is supported by the disclosures in the specification of the '127 patent. In fact, the '127 patent discloses that: “each end of the double-stranded target nucleic acid molecule is *ligated* to an SMI adaptor molecule. The double-stranded target nucleic acid complex is *then amplified* by a method known in the art (e.g., a PCR or non-PCR method known in the art), resulting in a set of uniquely labeled, amplified SMI-target nucleic acid products. These products are *then sequenced*” EX1001, 15:27-39 (emphasis added).

55. Moreover, Figure 1 also depicts PCR amplification occurring after adapter ligation step but prior to “massively parallel sequencing.” EX1001, Fig. 1. In addition, the specification further discloses the steps of “ligating” target DNA molecules to adapters, “amplifying” the adapter-DNA molecules, and then “sequencing the amplified ... products.” EX1001, 3:21-27.

56. Based on the plain language of the claims and the disclosures in the specification, a POSA would understand that (i) the claimed methods require that the amplification step occurs after adapter attachment, but before the sequencing step; and (ii) the sequencing step requires sequencing the amplified adapter-DNA products from the amplification step.

57. I also understand that the Board agrees with this construction of “amplification” as it is used in the claims. In the Decision on Institution, the Board stated the following:

Based on the current record, we find *Patent Owner's argument concerning the sequential order of the steps is supported by the claim language and the disclosures in the specification*. See, e.g., Ex. 1001, 37:31–52 (claim 1), 39:56–40:25 (claim 22), 3:21–27, 15:27–34, Fig. 1. Therefore, we agree with Patent Owner that “(i) the claimed methods require that the amplification step occurs after adapter attachment; but before the sequencing step; and (ii) the sequencing step requires sequencing the amplified adapter-DNA products from the amplification step.”

Paper 14 (D.I.), 10 (emphasis added).

58. The Board further stated that it agreed with “Patent Owner that in claim 1, the amplifying step (b) occurs after the adapter-attaching step (a) and before the sequencing step (c).” Paper 14 (D.I.), 16.

59. Thus, a POSA would understand from the plain language of the claims and the specification that the claimed methods recite amplification as a discrete and separate step after adapter attachment but before the sequencing step.

B. “First and second strand sequence reads”

60. Claim 1 recites “sequencing a plurality of first and second strand copies to obtain *first and second strand sequence reads* for at least a portion of the adapter-DNA molecules.” EX1001, claim 1. Claims 2-21 depend from claim 1 and thus also include this limitation. EX1001, claims 2-4, 16, 18, 19. A POSA reading this claim would understand that a “sequence read” refers to a contiguous set of

DNA nucleotides that correspond to the output of a DNA sequencing reaction, such as the sequencing-by-synthesis process depicted in Figure 1. EX1001, Fig. 1. In other words, a POSA would understand “first and second strand sequence reads” to mean a distinct sequence read of the first strand and a separate, distinct sequence read of the second strand.

61. Claim 1 recites sequencing the first and second strand copies to obtain first and second strand sequence *reads*, which indicates multiple, distinct sequencing reactions, each of which results in a sequence read, as opposed to a single sequencing reaction that produces a single sequence read containing multiple subparts. A POSA would understand that the claim would have recited “subreads” if it were directed to obtaining multiple subparts of a single sequence read from a single sequencing reaction. However, the claims do not recite obtaining subreads.

62. The specification supports the plain language of the claims. Indeed, Figure 3 of the '127 patent shows “*sequence reads* (brown) sharing a unique set of SMI tags being grouped into paired families with members having strand identifiers in either the $\alpha\beta$ or $\beta\alpha$ orientation.” EX1001, 4:12-17 (emphasis added). Moreover, the specification also states that “[w]hen these molecules are amplified with PCR and sequenced, they will yield the following *sequence reads*,” just as the term appears in the claims. EX1001, 4:45-47. In addition, the specification also

discloses as an example two distinct reads, “Read 1” and “Read 2” being generated after PCR amplification. EX1001, 9:5-9. The specification goes further to state that the “sequences of the two duplex strands seen in the two sequence reads [Read 1 and Read 2] may then be compared, and sequence information and mutations will be scored only if the sequence at a given position matches in both of the reads.” EX1001, 9:10-13.

63. Disclosures in Travers 2010 also further support this construction. Travers 2010 refers to the sense and antisense target DNA sequences as *subreads*, which are distinct from the “raw read sequence” or “raw read” that comprises all of the subreads physically linked together. EX1021, 2. As shown below, Travers 2010 refers to these individual sense and antisense sequences as subreads instead of sequence reads.

The first step is to classify the different regions in the raw circular molecule reads into *adapter and insert subreads*. This is done by *aligning a raw read sequence* to the known adapter sequence and a putative reference sequence of the insert regions. *Once all subreads are identified*, we test at each base position whether the subreads are generated by the putative reference sequence of the template or some variation of that sequence

EX1021, 2 (emphasis added).

64. Travers 2010 also discloses “align[ing] all the insert subreads

identified from a single-molecule raw read” and calling “the set of called bases corresponding to the insert region a ‘subread.’” EX1021, 2, 5. As such, a POSA would understand from Travers 2010 that the sense and antisense target DNA sequences are considered subreads, which altogether make up a raw read or a raw sequence read. Moreover, Eid 2009 refers to a sequence “read” as comprising repeating copies of the template sequences, rather than separate and distinct reads. EX2001, 4. In addition, Travers 2010 discloses that “[t]o detect the potential single-nucleotide polymorphism at a given location of the template, we *align all the insert subreads* identified from a single-molecule *raw read* to four different sequences representing the four possible SNP candidates using a probabilistic sequence aligner.” EX1021, 2. Moreover, Travers 2010 teaches that “[w]e call the set of called bases corresponding to the insert region a *subread*.” EX1021, 5.

65. Thus, a POSA would understand from the claim language, the specification, and the extrinsic evidence that the “first and second strand sequence reads” refer to distinct sequence reads of the first and second strand, and not subreads.

IX. LEGAL BASIS FOR MY ANALYSIS

A. Obviousness

66. I understand that a claim is invalid for obviousness if the differences between the claimed invention and the asserted prior art are such that the subject

matter as a whole would have been obvious to an ordinarily skilled artisan at the time the invention was made. Similarly, a claim is invalid for obviousness if two or more prior-art references in combination disclose, expressly or inherently, every claim limitation so as to render the claim, as a whole, obvious to a POSA at the time the invention was made.

67. I understand that obviousness is ultimately a question of law based on a determination of a number of factual issues. Those factual issues relate to: (1) the scope and content of the prior art, (2) the differences between the prior art and the claimed invention as a whole, (3) the level of ordinary skill in the art at the time the invention was made, and (4) objective secondary considerations that reflect, for example, the contemporaneous response to the invention at the time, such as commercial success, long-felt need, failure of others, industry-wide praise, and unexpected results.

68. I understand that care must be taken to avoid the impermissible use of hindsight in an obviousness analysis. I understand that it is improper to use the invention as a plan or template for hindsight reconstruction of bits and pieces of the prior art to form the invention.

69. It is my further understanding that a patent claim composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. There must be a reason why it would

have been obvious to modify the prior art to arrive at the claimed invention.

Counsel has informed me that the patent challenger has the burden to show that a POSA in the relevant field had a reason to combine the elements in the manner claimed when asserting obviousness in view of a combination of references.

70. I understand that there is no suggestion or motivation to make a modification to a prior-art reference if the proposed modification would render the prior-art invention unsatisfactory for its intended purpose. I also understand that an obviousness allegation cannot be supported by a combination of references that would require a substantial reconstruction and redesign of the elements shown in the primary reference or a change in the basic principle under which the primary reference was designed to operate.

71. I understand that where there is a reason to modify or combine the prior art to achieve the claimed invention, there must also be a reasonable expectation of success in doing so. I also understand that conclusory statements lacking factual substantiation are insufficient for evaluating reasonable expectation of success as part of an obviousness determination. Instead, I understand that the Petitioner must articulate a basis on which it concludes that it would have been obvious to make the claimed invention.

72. I have been informed that objective factors concerning non-obviousness, also known as “secondary considerations,” may be probative of a

claim's obviousness. I include my understanding of the specific legal principles relating to each secondary consideration in my discussion below. I have also been informed that, in order for the secondary consideration to have weight, there must be a nexus between the evidence of the alleged secondary consideration and the claim as a whole. I understand that, without nexus, evidence of secondary considerations does not support a finding of non-obviousness. I further understand that evidence of secondary considerations will have no weight if the evidence is related to an unclaimed feature or features found in the prior art.

73. I understand that a patent holder can establish nexus in two ways: (1) establishing a rebuttable presumption of nexus by showing that a product to which evidence exists both embodies the claimed features and is coextensive with the patented invention or (2) demonstrating that the presented evidence is a direct result of the unique characteristics of the claimed invention.

B. Enablement

74. I understand that to render a claim obvious, a POSA must have a reasonable expectation of success in making the proposed combination. I understand that enablement is a related concept. In order to be enabling, I understand that the prior art must allow a POSA to make and use the relied upon portion of the disclosure without undue experimentation. I understand that the question of undue experimentation involves consideration of a number of factors,

including: (i) the quantity of experimentation necessary, (ii) the amount of direction or guidance presented, (iii) the presence or absence of working examples, (iv) the nature of the invention, (v) the state of the prior art, (vi) the relative skill of those in the art, (vii) the predictability or unpredictability of the art, and (viii) the breadth of the claims. I understand that whether experimentation is undue is based on the level of skill in the art as of the effective filing date of the application on which the patents-in-suit claim priority.

X. DR. QUACKENBUSH FAILS TO ADDRESS ALL LIMITATIONS OF THE CHALLENGED CLAIMS.

A. Travers '075 and Travers 2010 do not teach first strand sequence reads and second strand sequence reads, as recited in claims 1-21.

75. As discussed above in Section VIII.B, Claim 1 step (c) requires obtaining “first and second strand sequence reads.” EX1001, claim 1. Based on the discussion above, a POSA would understand that first strand sequence reads and second strand sequence reads are separate, distinct sequence reads. A POSA would also understand that the first and second strand sequence reads recited in claims 1-21 are also separate, distinct sequence reads, representing distinct sequencing reactions. EX1001, claims 1-21.

76. Dr. Quackenbush has not sufficiently shown that Travers 2010 or Travers '075 discloses this limitation of the claims. A POSA would have understood that Travers 2010's disclosure of “sequence reads” are not separate and

distinct sequence reads, but rather subreads *physically linked together* in a single read produced from a single sequencing reaction. These sequence reads are not separate sequence reads. Indeed, Travers 2010 refers to the sense and antisense target DNA sequences as *subreads*, which are distinct from the “raw read sequence” or “raw read” that comprises all of the subreads from sequences physically linked together and sequenced in the same sequencing reaction:

The first step is to classify the different regions in the raw circular molecule reads into *adapter and insert subreads*. This is done by *aligning a raw read sequence* to the known adapter sequence and a putative reference sequence of the insert regions. *Once all subreads are identified*, we test at each base position whether the subreads are generated by the putative reference sequence of the template or some variation of that sequence.

EX1021, 2 (emphasis added).

77. In addition, Travers 2010 discloses that “[t]o detect the potential single-nucleotide polymorphism at a given location of the template, we *align all the insert subreads* identified from a single-molecule *raw read* to four different sequences representing the four possible SNP candidates using a probabilistic sequence aligner.” EX1021, 2. Moreover, Travers 2010 teaches that “[w]e call the set of called bases corresponding to the insert region a *subread*.” EX1021, 5.

78. This understanding is further confirmed in Eid 2009, which is

considered to be PacBio's pivotal SMRT sequencing paper. Specifically, Eid 2009 refers to a sequence "read" as comprising repeating copies of the target sequences, rather than separate and distinct reads. EX2001, 4. Eid 2009 specifically states that "a *representative read* (Fig. 3B) showed the expected continuous signature sequence *pattern of alternating periods* of A555-dCTP and A647-dGTP pulses." EX2001, 4 (emphasis added). Thus, a POSA would understand that Eid 2009 was not considering a sequence "read" to mean separate and distinct reads from different sequencing reactions.

79. As discussed above, claim 1 and its dependent claims require obtaining separate and distinct sequence reads for the first strand sequence read and the second strand sequence read and "confirming the presence of at least one sequence read derived from each of the original first and second strands," respectively. This is different from the single sequence read comprising subreads that are linked together disclosed in Travers '075 and Travers 2010. Instead of addressing this part of the claims, Dr. Quackenbush merely argues that "Travers discloses that either SMRTbell templates, or their replication products (i.e., concatemers), may be sequenced using the disclosed template-dependent sequencing process." EX1002, ¶81. Neither reference discusses separate sequence reads derived from each of the original first and second strands as claimed. As such, Dr. Quackenbush does not address the specific limitations regarding first and

second strand sequence reads recited in these claims.

80. Thus, this claimed element is missing from the applied art. And Dr. Quackenbush has not demonstrated that claims 1-21 are obvious.

B. Travers '075 and Travers 2010 do not teach grouping the sequence reads into families based on barcode sequences and DNA fragment-specific information as recited in claims 22-30.

81. Claim 22 step (d) recites the following: “for at least some of the double-stranded adapter-DNA molecules in the library— grouping the strand sequence reads into families based on i) the barcode sequence, *and* ii) DNA fragment-specific information.” EX1001, claim 22. Since claims 23-30 depend from claim 22, they each include this claim limitation as well. Travers '075 and Travers 2010 do not teach the step of grouping the sequence reads into families based on barcode sequences and DNA fragment-specific information, as claimed in claims 22-30.

82. Dr. Quackenbush argues that paragraph 100 of Travers '075 discloses “the use of ‘oligonucleotide tags’ or barcodes to ‘pool[data] for analysis.’” EX1002, ¶113. But Dr. Quackenbush cherry-picks quotations from Travers '075, which places the disclosures entirely out of context. Neither Travers 2010 nor Travers '075 discloses the step of grouping the sequence reads into families based on barcode sequences and DNA fragment-specific information, as claimed in claims 22-30.

83. In the proper context, Travers '075 (EX1018) discloses adding sample barcodes (also known in the art as *index barcodes*) to track sample origins, not grouping the strands into families based on barcode sequence and DNA fragment-specific information. Specifically, Travers '075 teaches the following:

In particular, one can utilize connecting oligonucleotides in the template constructs that include sequence markers like a *barcode*, to indicate the origin of a given template sample. Different template samples including distinguishable connecting oligonucleotide tags could then be pooled for analysis in a single sequencing process. Sequence data derived from individual templates is then attributable to the originating sample preparation process through the identification of the connecting oligonucleotide tag.

EX1018, ¶[0100] (emphasis added).

84. Travers '075 utilizes “pooling” of indexed barcoded samples to track the sample source. The use of indexed barcoded samples is very different from the claimed step of grouping the strands of sequence reads into families based on barcode and DNA fragment-specific information. Indexing barcodes or sample barcodes are short, unique sequences of DNA added to all molecules from a specific *sample* so samples can be pooled, then processed and sequenced in parallel with each resulting sequence containing information to determine the source material (starting sample). EX2034, 4. Pooling of indexed barcoded samples is

very different from molecular barcoding as recited in claims 22-30, where each target molecule has a barcode sequence so that reads from each specific target molecule can be identified. Moreover, claims 22-30 require grouping strand sequence reads based on the barcode sequence and DNA fragment-specific information, which is not disclosed in Travers '075.

85. I also understand that Guardant has argued that index sequences are different from the molecular barcodes used in the claimed step of grouping the strands of sequence reads into families based on barcode and DNA fragment-specific information. In a European Opposition proceeding involving one of Guardant's European patents, Guardant argued that index sequences are "used to permit multiplexing of several different samples in a single sequencing experiment," and that a POSA would *not* "consider using the index sequence for collapsing sequences into sets [] or 'UID [unique identifier] families.'" EX2019, 18. In other words, Guardant argued that index barcodes are different from molecular barcodes. I agree.

86. Dr. Quackenbush also fails to explain how Travers '075 or Travers 2010 teaches the requirement in claims 22-30 to use both the barcode sequence and "DNA fragment-specific information" to group the sequence reads into families. Dr. Quackenbush argues that "Travers expressly describes determining consensus sequences *based on assembled (i.e., grouped)* redundant sequence reads," but does

not explain *how* Travers '075 and Travers 2010 “group” subreads. EX1002, ¶113.

A POSA would have understood that the subreads in Travers '075 and Travers 2010 are only “grouped” because they are physically connected to one another. Second, the sample barcodes disclosed in Travers '075 must be identical for all template molecules within a sample, and therefore are not fragment-specific by definition. As such, Dr. Quackenbush has not explained how the subreads in either Travers '075 or Travers 2010 are grouped based *on DNA fragment-specific information* (or barcode sequence), as recited in claims 22-30.

87. Thus, Travers '075 and Travers 2010 do not teach the grouping step limitation of claims 22-30.

C. Travers '075 and Travers 2010 do not teach distinguishing the first and second strands of an original adapter-DNA molecule, as recited in claim 19.

88. Claim 19 recites: “The *method* of claim 1, wherein the *first strand sequence reads* derived from a particular original adapter-DNA molecule can be *differentiated from the second strand* sequence reads derived from the same particular original adapter-DNA molecule *at least in part by an asymmetry of the asymmetrical double-stranded adapter-DNA molecules.*”² EX1001, claim 19;

² I understand that a Certificate of Correction was issued on March 9, 2021 to correct claim 19. EX2020, 482.

EX2020, 482.

89. A POSA would understand that the method of claim 19 recites *using* the asymmetric nature of the adapters to differentiate the first strand sequence read and second strand sequence read. Indeed, the specification of the '127 patent discloses that the asymmetric nature of the adapters are used to differentiate the two strands:

By virtue of the asymmetric nature of adapted fragments, two types of PCR products are produced from each capture event. Those derived from one strand will have the α SMI sequence adjacent to flow-cell sequence 1 and the β SMI sequence adjacent to flow cell sequence 2. PCR products originating from the complementary strand are labeled reciprocally.

EX1001, 3:64-4:3 (emphasis added).

90. Dr. Quackenbush argues that Travers '075 and Travers 2010 teach or suggest every element of claim 19 because they allegedly teach “that the asymmetry of adapter-DNA molecules imbued by the hairpin linker *allows* the first and second strands of an original adapter-DNA molecules to be distinguished.”

EX1002, ¶¶171-173. I disagree. Travers '075 and Travers 2010 do not disclose or even suggest using the asymmetry of adapters to distinguish between first and second strands, as claimed in claim 19.

91. For example, Dr. Quackenbush relies upon the disclosures in

paragraph 54 of Travers '075 to support the allegation that the asymmetry of the adapters were used to distinguish between the top and bottom strands. EX1002, ¶¶171-172. However, paragraph 54 of Travers '075 does not disclose using the *asymmetric nature* of the adapter-DNA fragments to distinguish the first and second strand sequence reads. Rather, Travers '075 discloses identifying only the presence of adapter sequences as landmarks or “registration sequences” that mark the portion of the sequence read where one strand ends and the next strand begins. EX1018, ¶[0054]. A POSA would understand that this disclosure in paragraph 54 is merely indicating that the linking segment can be used to identify the order of sequence segments (e.g., target-adapter-target-adapter, etc.). This is different from using the asymmetrical configuration recited in claim 19 to differentiate the two strands. A POSA would understand that the adapter sequences in Travers '075 and Travers 2010 do not need to provide any asymmetry to the adapter-DNA molecules.

92. In fact, Travers '075 states in paragraph 54 that “the presence of linking segment 206 also provides an opportunity to provide *a registration sequence* that permits the identification of *when one segment, e.g., 202, is completed and the other begins*, e.g., 204.” EX1018, ¶[0054]. In addition, Travers '075 discloses that the “registration sequence indicat[es] a point in the sequence ... [that] transitions from the sense to the antisense strands.” EX1018, ¶[0054].

Travers '075 also teaches that “because the connecting oligonucleotide is known or knowable, one can readily exploit this pre-known sequence in aligning long strings of sequence data from such template or templates, e.g., *as a landmark or registration sequence.*” EX1018, ¶[0098] (emphasis added). These disclosures only relate to the order of sequence segments in the sequence read and have nothing to do with adapter asymmetry.

93. Dr. Quackenbush also cites to Figures 1 and 4 of Travers 2010 for support that claim 19 is obvious. EX1002, ¶¶172-173 (citing EX1021, Figs. 1, 4). However, Figure 1 in Travers 2010 says nothing about using the asymmetric nature of adapters to distinguish strands. Figure 1 only discloses a schematic of the SMRTbell template without any further disclosure regarding how the asymmetric nature of the hairpins are used to differentiate the first and second strand of the template molecule. EX1021, Fig. 1. And Travers 2010's Figure 4 only discloses using the adapter sequences as landmarks to identify when one target strand ends and the next target strand begins. In fact, Travers 2010's Figure 4 depicts single-molecule traces of both the sense and antisense strands, and notes that the pulses within the sequencing traces are colored according to location within the template, with blue corresponding to the sense strand, orange corresponding to the antisense strand, and light blue and green corresponding to the hairpin adapters. EX1021, Fig. 4. A POSA would not understand this disclosure to be teaching using the

asymmetric nature of the hairpins to differentiate the strands, as required by claim 19.

94. Thus, nothing in Travers '075 or Travers 2010 discloses using the asymmetric nature of the adapter-DNA molecules to distinguish strands, as claimed in claim 19. In addition, claim 19 is a method claim, and Dr. Quackenbush has not sufficiently shown that Travers '075 and Travers 2010 teach *why* or *how* to differentiate the first and the second strand using the asymmetric nature of the hairpin adapters. All Dr. Quackenbush states is that “the asymmetry of adapter-DNA molecules imbued by the hairpin linker *allows* the first and second strands of an original adapter-DNA molecules to be distinguished.” EX1002, ¶172.

95. Thus, Dr. Quackenbush has not sufficiently shown that Travers '075 and Travers 2010 teach all elements of claim 19.

XI. A POSA WOULD HAVE NO REASON TO COMBINE TRAVERS '075'S ROLLING CIRCLE REPLICATION EMBODIMENT WITH THE SEQUENCING APPLICATIONS OF TRAVERS 2010.

A. Dr. Quackenbush fails to explain *why* or *how* a POSA would have combined Travers '075's rolling circle replication embodiment with the SMRT sequencing applications disclosed in Travers 2010.

96. As discussed above, the PacBio SMRT sequencing workflow is different from the claimed methods, which require a separate amplification step prior to the sequencing step. Dr. Quackenbush first argues that paragraph 83 of Travers '075 teaches that the SMRTbell template molecules are “subjected to a

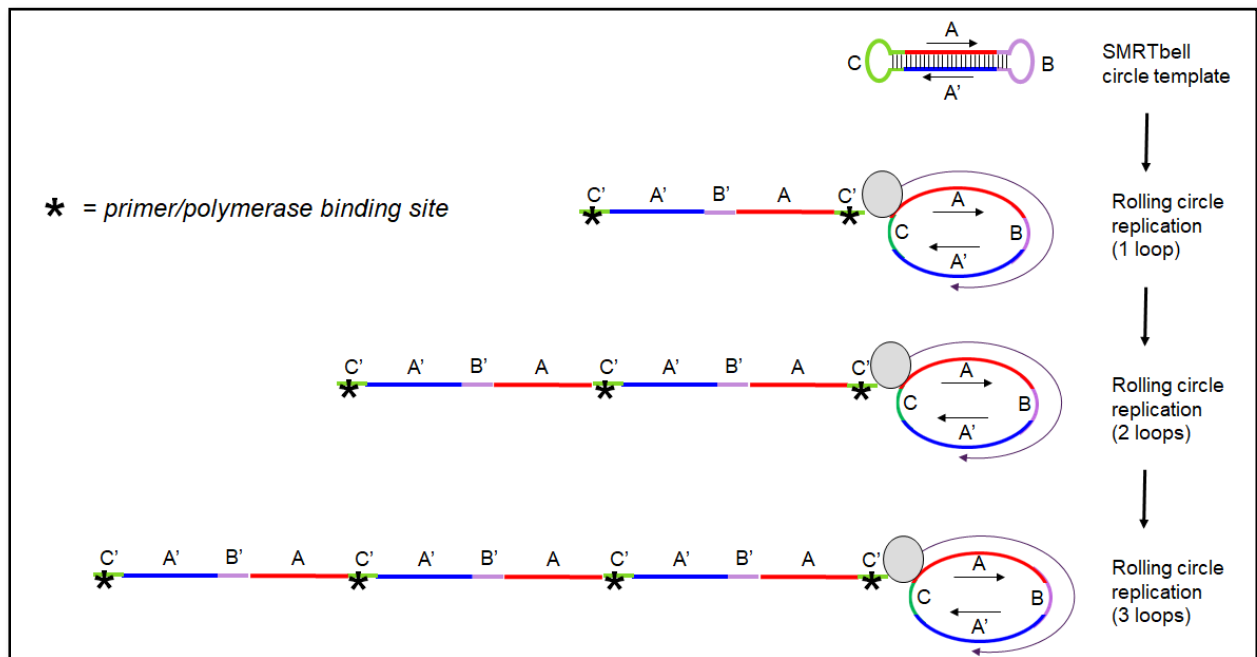
polymerase mediated sequencing process.” EX1002, ¶78. This disclosure in Travers ’075 does not teach a separate template-amplification step required by the claimed methods. Instead, this reference to a “polymerase mediated sequencing process” relates to the well-known “sequencing by synthesis” process of SMRT sequencing. EX2003, 5; EX2004, 8; EX2021, 1, 6; EX2022, 1. A POSA would have recognized that Travers ’075 was referring to this well-understood sequencing by synthesis process in paragraph 83, and was not disclosing a separate template-amplification step.

97. Next, Dr. Quackenbush argues that a rolling circle replication embodiment taught in Travers ’075 combined with the SMRT sequencing applications of Travers 2010 teaches the amplification of template molecules prior to sequencing. Specifically, Dr. Quackenbush argues that Travers ’075 explains that “rolling circle replication” of SMRTbell templates produces concatemer molecules comprising “repeating copies of both the sense and antisense strands of the originating double stranded segment,” and that these replicated products may “be employed directly as template molecules in a template dependent sequencing process.” EX1002, ¶¶57, 77-82, 105-110, 114-115, 151-153, 177; EX1018, ¶[0122].

98. As seen in the figure below, I have depicted exemplary products of Travers ’075’s rolling circle replication embodiment. According to Dr.

Quackenbush, the product of such a rolling circle replication step is a long single-stranded concatemer that features a number of repeating copies of both the sense and antisense strands of the template, but also the hairpin sequences as well.

EX1002, ¶¶77-82. As shown below, the concatemer sequence is generated via rolling circle replication by the DNA polymerase, indicated by the gray oval, binding to the primer sequence on the green hairpin (C) and then synthesizing the sequences in a clockwise fashion with the sense strand (A), the purple hairpin sequence (B), the antisense strand (B), and then the green hairpin sequence in a continuous loop. As shown below, the replicated concatemer product increases in length with the number of loops around the SMRTbell template.



99. I understand that the Board instituted this case by relying on Dr.

Quackenbush's arguments that the disclosed circular molecules in Travers '075 and Travers 2010 may be amplified by rolling circle replication to produce concatemer molecules, which could then be subject to SMRT sequencing. Paper 14 (D.I.), 19. Specifically, the Board noted that the template molecules disclosed in Travers '075 and Travers 2010 "may be subject to SMRT sequencing directly, or they may be amplified to produce concatemer molecules, which are then subject to SMRT sequencing." Paper 14 (D.I.), 19. For the detailed reasons I provide below, I disagree that a POSA would have considered combining Travers '075's rolling circle replication embodiment with the SMRT sequencing applications disclosed in Travers 2010. Moreover, even if a POSA had attempted to do so, a POSA would not have expected to achieve useful sequencing reads.

100. Indeed, Dr. Quackenbush provides no explanation of *why* a POSA would have been motivated to combine Travers '075's rolling circle replication embodiment with the SMRT sequencing applications disclosed in Travers 2010. Moreover, as discussed in detail below, there are at least *four* critical problems with Travers '075's rolling circle replication embodiment. As explained below, these four problems would have rendered Travers '075's rolling circle replication embodiment unworkable and requiring undue experimentation. Thus, a POSA would have been dissuaded from considering such an embodiment.

B. A POSA would not have been motivated to modify the PacBio SMRT sequencing workflow of Travers 2010, which does not contain a template amplification step, with Travers '075's rolling circle replication embodiment.

1. Numerous references both in the art and published after March 20, 2012, taught that the SMRT sequencing of Travers '075 and Travers 2010 did not require a template-amplification step.

101. Before March 20, 2012, the art was brimming with references teaching that the PacBio SMRT sequencing workflow discussed in Travers '075 and Travers 2010 does not use a SMRTbell template-amplification step. It was well known that not only did the Travers' SMRT sequencing method not rely on an amplification step, but this was also considered an important advantage of the method. Indeed, Travers 2010 expressly discloses that “this protocol for template preparation requires a minimal number of steps and *does not depend on amplification.*” EX1021, 5 (emphasis added). Moreover, Travers '075 states that “the constructs of the invention are described *primarily, and preferably*, for use *directly as templates* for, e.g., sequencing applications.” EX1018, ¶[0122] (emphasis added).

102. For example, Niedringhaus 2010 reported that “[Travers '075 and Travers 2010's] [s]ample preparation time is decreased since *template amplification is not needed.*” EX2002, 4 (emphasis added). Niedringhaus 2010 also disclosed that an “advantage” of the PacBio SMRT sequencing workflow is that

there is “*no amplification* of sequencing fragments.” EX2002, Table 2 (emphasis added). Likewise, Schadt 2010 taught that “*SMRT sequencing does not require routine PCR amplification* needed by most SGS systems, thereby *avoiding systematic amplification bias*.” EX2023, 7. Schadt 2010 further taught that amplification bias can lead to “over-representation of some sequences and under-representation of others,” and that the “amplification process can introduce errors in the template sequence.” EX2023, 2, 12. In addition, Schadt teaches that these limitations associated with amplification steps are “pathologies,” and discloses that as a result, “neither the sequences nor the frequencies with which they appear are always faithfully preserved.” EX2023, 2. A POSA would understand that these represented inherent challenges associated with amplification steps.

103. Likewise, Thompson 2011 described the method of Travers 2010 as “creating a circular template (called SMRTbell for single molecule real time), and then repeatedly sequencing the same molecule.” EX2003, 5. Thus, Thompson disclosed that the method of Travers ’075 and Travers 2010 involves directly sequencing the template without amplification. Liu 2011 similarly taught that “[t]he key difference between NGS and single-molecular sequencing technology [e.g., the SMRT™ sequencing of Travers ’075 and Travers 2010] is that, in NGS, DNA molecules have to be amplified before sequencing, while *the latter enables us to sequence a single DNA molecule; thus, avoid the time, error and cost*

associated with sample preparation and amplification.” EX2024, 2 (emphasis added). Liu 2011 goes on to state that “[t]he sample needed for running SMRT™ DNA sequencing is ~1 ng of DNA, which suggests that *no PCR amplification is needed*. This also *lowers the risk of having systematic amplification bias.*” EX2024, 2.

104. Numerous contemporaneous references also taught that the sequencing method disclosed in Travers ’075 and Travers 2010 did not utilize an amplification step after adapter ligation, but before or during sequencing. For example, Stranneheim 2012 characterized the “sample preparation” process disclosed in Travers ’075 and Travers 2010 as having “*no amplification.*” EX2004, 4, Table 1 (emphasis added). Quail 2012 reported a comparison of different sequencing methods and described the SMRT sequencing methods of Travers ’075 and Travers 2010 as having “an *amplification free* workflow,” and further referred to the “*amplification-free* library prep of PacBio.” EX2005, 2, 9 (emphasis added). Egan 2012 similarly reported comparing SMRT sequencing with other sequencing methods, noting that one of the “pros” of SMRT sequencing was that “*no template amplification [is] needed.*” EX2006, Table 1 (emphasis added).

105. Koren 2012 also taught that SMRT sequencing “aims to address the problems outlined above by *requiring no amplification* and reducing compositional bias.” EX2025, 1 (emphasis added) (citations omitted). Ribeiro 2012 also agreed

that “the Pacific Biosciences data are generated without amplification and thus could provide coverage in regions that are underrepresented or absent in Illumina data due to amplification bias in the sample preparation.” EX2054, 1.

106. References published after March 20, 2012 also taught that the SMRT™ sequencing of Travers ’075 and Travers 2010 did not utilize an intervening amplification step prior to or during sequencing. Fichot 2013 explained that “[u]nlike Illumina and 454, *the library preparation for [the SMRT sequencing in Travers ’075 and Travers 2010] does not include an amplification step*, which avoids this as a potential sequencer-based error source.” EX2022, Abstract, 1, 4 (emphasis added).

107. I understand from reviewing the Decision on Institution in this case that the Board agrees that no amplification is required in the SMRT sequencing methods disclosed in Travers ’075 and Travers 2010. Indeed, the Board stated that “we agree with Patent Owner that the *Travers references describe SMRT sequencing, which does not require amplification before sequencing*, as the primary and preferred embodiment.” Paper 14 (D.I.), 18.

108. Thus, references published before and after March 20, 2012 consistently taught that the SMRT sequencing method of Travers ’075 and Travers 2010 relied upon by Dr. Quackenbush does not require any SMRTbell template-amplification step after adapter attachment, but before or during sequencing the

SMRTbell templates. Moreover, these references taught that the absence of a SMRTbell template-amplification step in the SMRT sequencing method of Travers '075 and Travers 2010 was a notable feature and an advantage of the workflow that differentiated it from other sequencing methods. If a POSA were to modify the SMRT sequencing of Travers '075 and Travers 2010 by adding a rolling circle replication step, such a step would have destroyed this key advantage of the circular consensus sequencing approach disclosed in Travers '075 and Travers 2010.

109. Dr. Quackenbush argues that Travers '075 describes amplification of template molecules using rolling circle replication prior to redundant sequencing and that replicated concatemer products *may then* be employed as template molecules in a sequencing-dependent process without providing any details as to why a POSA would have been motivated to do so. However, Travers '075 does not provide any details on how to successfully incorporate such a rolling circle replication embodiment into the SMRT sequencing workflow.

110. As I discuss in detail below, a POSA would *not* have sought to discard the established SMRT workflow to implement an unnecessary and problematic rolling circle replication step mentioned in Travers '075. Indeed, Dr. Quackenbush has not shown that a POSA would have desired to modify the SMRT sequencing workflow disclosed in Travers '075 and Travers 2010 with a rolling circle

replication step. If a POSA were to modify the SMRT sequencing of Travers '075 and Travers 2010 by adding a rolling circle replication step, such a step would have destroyed the entire purpose of the circular consensus sequencing approach described in Travers '075 and Travers 2010, as I elaborate below. Moreover, Travers '075 expressly disclosed that its preference is to use its circular templates directly in sequencing *without* any template-amplification step. EX1018, ¶[0122].

111. For starters, a POSA would not have wanted to include an additional amplification step into the SMRT sequencing workflow because of the known error rates associated with DNA polymerases. For example, the polymerases used in rolling circle replication reactions would certainly introduce errors in the amplification process. EX2017, 2; EX2068, 6. As such, a POSA would have known that introducing an unnecessary amplification step into the amplification-free SMRT sequencing workflow, as Dr. Quackenbush proposes, would only make SMRT sequencing worse by generating more sequence errors into the already error-ridden SMRT sequencing workflow. Indeed, SMRT sequencing was already known in the art for having the “highest raw error rates of NGS methods.” EX2006, 5.

112. Most importantly, a POSA would have understood that the replicated concatemer products from rolling circle replication would be fundamentally incompatible with the SMRT sequencing method disclosed in Travers '075 and

Travers 2010 using ZMWs. Specifically, a POSA would have expected implementing a rolling circle replication step in the SMRT sequencing workflow to cause numerous problems, including varying rolling circle concatemer product sizes, DNA diffusion issues, and geometrical constraints of the zero mode waveguides described in Travers '075 and Travers 2010, making the products unsuitable templates for PacBio sequencing.

C. A POSA would have understood that rolling circle replication is incompatible with the SMRT sequencing method of Travers '075 and Travers 2010 on a zero mode waveguide (ZMW) chip.

1. The SMRT sequencing workflow had a high error rate, which required using circle sequencing to re-sequence the template multiple times.

113. As discussed above, in the SMRT sequencing methods of Travers '075 and Travers 2010, single molecule circular templates, called SMRTbells, are added to the ZMW array. EX1021, 3-5; EX1018, ¶¶[0141], [0146]. These small molecules diffuse through the medium and into the ZMW wells, where they can be sequenced multiple times. EX1018, ¶¶[0053], [0055], [0058], [0141], [0146]. A POSA would have understood that SMRT sequencing with circular templates required resequencing the same template repeatedly with circular consensus sequencing to provide a sufficient level of redundancy for error correction. This was because it was well known that the SMRT™ sequencing of Travers '075 and Travers 2010 was prone to having high error rates—the highest of all other next-

generation sequencing platforms. In fact, Stanneheim states that the “SMRT sequencing approach has great potential with unique features and fast sequencing reaction runs, but suffers from raw error rates exceeding 10%, largely due to indels.” EX2004, 9.

114. Glenn 2011 reported an error rate of 16%, while Neidringhaus 2010 and Thompson 2011 reported raw error rates of 17-19% or 15-20%, respectively. EX2034, Table 3, 9; EX2002, 5; EX2003, 5. Thompson 2011 disclosed that SMRT sequencing’s error rate was “significantly *higher than with any other current sequencing technology*, creating challenges in using the data for some applications, such as variant detection.” EX2003, 5 (emphasis added). Thompson 2011 further noted that “the high raw error rate can be overcome by ligating a hairpin oligonucleotide to each end of the DNA, creating a circular template (called SMRTbell for single molecule real time), and then repeatedly sequencing the same molecule.” EX2003, 5. Thompson 2011 also disclosed that this procedure of repeatedly sequencing the same molecule “works when the molecules are relatively short but it cannot be used with long reads, so those retain the high raw error rate.” EX2003, 5. Similarly, Egan 2012 teaches that SMRT sequencing has one of the highest error rates that “*requires* multiple rounds of sequencing to reduce.” EX2006, 4-5, Table 1 (emphasis added).

115. Shendure 2011 taught that SMRT sequencing’s “reads have, on

average, 8% to 10% error rate and, to increase the accuracy of sequencing, *multiple passes are needed*, which considerably decreases throughput.” EX2032, 12.

Ozsolak 2012 taught that SMRT’s error rate of 15-20% was the “highest among the commercially available sequencing technologies today” and that this error rate was “not expected to improve significantly in the near future.” EX2035, 6. Mardis 2013 taught that “the [SMRT sequencing] error rate is approximately 15% (15 bases per 100), yet the consensus error rate obtained from multiple passes on a single molecule is >97%.” EX2033, 14. Thus, a POSA would have been well aware of the high error rates associated with SMRT sequencing.

116. As I discussed above, it was also well known that the only way to overcome this persistently high error rate was to resequence the SMRTbell template over and over again to generate a consensus sequence. Indeed, Dr. Quackenbush cites to Travers 2010’s disclosure that the quality of the consensus sequence increases with the number of redundant reads of the molecule. EX1002, ¶¶129, 139, 143. Dr. Quackenbush stated that Travers 2010 explains that the quality of the consensus sequence can be “tuned” such that the consensus sequence allows for true variants to be distinguished from sequencing errors and that multiple reads of the same molecule increases the quality of the consensus sequence. EX1002, ¶¶129, 139, 143; EX1021, 6-7; EX1018, ¶¶[0007], [0053], [0089], [0142], [0143].

117. Travers 2010 also teaches that there is a tradeoff between the number of times a circular template can be sequenced and the empirical quality value (EQV), which is determined by the fraction of total base calls that are erroneous. For example, Travers 2010 teaches that unlike other systems, “the strategy of applying circular consensus sequencing to rare variant detection allows yield and read length to be exchanged for higher QV.” EX1021, 6. In other words, smaller circular SMRTbell templates can be re-sequenced many times to achieve a lower error rate and achieve a higher empirical quality value (EQV) in the sequencing data, which equates to a higher confidence in the data. However, larger templates, which cannot be re-sequenced as many times (if at all), result in a lower EQV and lower confidence. To illustrate this point, Travers 2010 discloses the following:

[C]onsider a system with an average read length of 1000 [nucleotides]. With a template of approximately 300 bp, an average of three reads can be obtained from the template. With a template size of approximately 250 bp, four reads can be obtained and with a template size of approximately 200 bp, five reads can be obtained. In this manner, *the choice of template size determines the number of reads that can be obtained from that template and therefore the final empirical QV (EQV) of the consensus data.*

EX1021, 6 (emphasis added). In sum, Travers 2010 teaches that the smaller the size of the template molecule, the larger the number of reads that can be obtained

from that template, and thus the better the quality of the consensus data. As discussed above, Travers 2010 teaches that multiple reads of the same molecule increases the quality of the consensus sequence. EX1021, 6-7; EX1018, ¶¶[0007], [0053], [0089], [0142], [0143].

118. Travers 2010 discloses this tradeoff in Figure 7 which depicts the unfiltered distribution of quality values as a function of the depth of coverage or the number of reads obtained on a template molecule. EX1021, Fig. 7. As depicted below, Travers 2010 teaches that the highest empirical quality value is achieved when the number of reads reaches 6x coverage depth, which is the depth achieved with three full loops around the circular SMRTbell template. EX1021, Fig. 7.

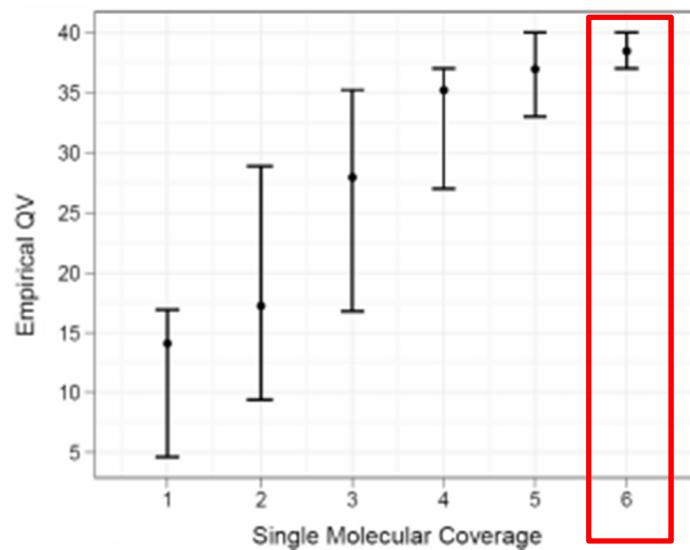


Figure 7. The unfiltered distributions of EQV as a function of single-molecule coverage (each full synthesis of a SMRTbell corresponds to 2× single-molecule coverage representing the forward and reverse strands). The upper and lower error bars represent the 75th and 25th quartile, respectively.

EX1021, Fig. 7 (annotated).

119. As indicated in the annotated Figure 7 above, the EQV at 6x molecular coverage was the highest with the error bars extending from 37-40. Travers 2010 discloses using “the phred-style quality value (QV)” to determine the quality value of the consensus base calls. EX1021, 5. A POSA would have understood that these QV scores are defined as a property that is logarithmically related to the base calling error probabilities. EX2055, 1. Indeed, Travers 2010 teaches that the QV was calculated as equivalent to “ $-10 \log_{10}$ ” or the “number of base call errors/total number of base calls.” EX1021, 5. A POSA would also have understood that since QV is a logarithmic property, then having a QV score of 10 is equivalent to the probability of an incorrect base call 1 in 10 times and a score of 20 being equal to a probability of an incorrect base call 1 in 100 times, and so on. EX2055, 1.

120. A POSA would understand the importance of obtaining reliable sequencing data with low error rates. For example, the diploid human genome consists of approximately 6.4 billion nucleotides. EX2066, Abstract. Therefore, for many sequencing applications, maintaining a low error rate is important for accurately identifying mutations.

121. Knowing this, a POSA reviewing the data in Figure 7 of Travers 2010 would have understood that the EQV or measured empirical quality values at 6x coverage depth ranged from 37-40, which means that the probability of an

incorrect base call was nearly 1 in 10,000. EX2055, 1. As such, a POSA would have desired to re-sequence the target DNA copies multiple times to reduce the error rate associated with SMRT sequencing and achieve a higher EQV, which is needed to generate useful sequencing data.

122. A POSA would have understood that combining Travers '075's rolling circle replication embodiment with the SMRT sequencing workflow disclosed in Travers '075 and Travers 2010 fundamentally changes the output. Instead of a single molecule circular SMRTbell template that is designed specifically for circular re-sequencing, a large, linear concatemer molecule is produced from rolling circle replication. As discussed below, Travers '075's rolling circle replication embodiment would have created critical problems when used in the SMRT sequencing applications of Travers 2010 because rolling circle replication produces a wide range of linear product sizes.

123. *First*, a POSA would expect that a Travers '075's rolling circle replication embodiment would form linear concatemers containing repeating copies of the sequencing primer and polymerase binding sites. As a result, a POSA would expect multiple polymerases to bind to multiple sequencing initiation sites. Given that the ZMW array used in the SMRT sequencing workflow can only sequence a single template with a single polymerase, Travers '075's linear concatemer products with multiple polymerase enzymes bound would produce

unreadable sequencing data.

124. *Second*, a POSA would have understood that the smaller-sized replication products would not possess enough copies of the target DNA required for effective SMRT sequencing. As a result, these products would be unsuitable for Travers 2010's SMRT sequencing applications. In addition, as discussed below, these smaller-sized replication products would not meet the claimed limitations of the '127 patent.

125. *Third*, a POSA would understand that the larger-sized replication products in Travers '075's rolling circle replication embodiment would be outcompeted by the smaller replication products for occupancy in the ZMWs for SMRT sequencing due to well-known DNA diffusion bias issues.

126. *Fourth*, a POSA would understand that the large linear concatemers would be expected to form complex secondary and tertiary structures, which would impede their ability to even fit within the narrow ZMW wells for SMRT sequencing.

127. Dr. Quackenbush does not address any of these problems that would not occur in the traditional circular SMRTbell template approach. As such, a POSA would not have had any motivation to toss aside the traditional SMRT sequencing workflow to implement Travers '075's rolling circle replication embodiment.

2. Problem 1: A POSA would have known that the DNA polymerase can bind at multiple start sites within concatemer templates preventing any useable sequencing data from being collected.

128. Dr. Quackenbush overlooked a fundamental problem with his proposed modification. A POSA would understand that the linear concatemer product of the Travers '075 rolling circle replication embodiment would have had multiple sequencing reaction start sites throughout the concatemer with at least one start site for each copy of the SMRTbell template in the concatemer. As a result, a POSA would also understand that multiple polymerase enzymes would bind to multiple different start sites, making the resulting template-polymerase complex unable to be sequenced on the ZMWs. This would have created a problem with the single-molecule design of the SMRT sequencing workflow of Travers '075 and Travers 2010.

129. In the SMRT sequencing workflow of Travers '075 and Travers 2010, a circular DNA template with *one and only one* sequencing primer binding site is pre-incubated with sequencing primer and polymerase. EX1021, 2; EX2001, 3. These components form a 1:1:1 primer-template-polymerase complex. EX1021, 2; EX2001, 3. After this complex is formed, it is then distributed and immobilized at the bottom of a ZMW well for sequencing. EX1021, 2; EX2001, 3. Indeed, Travers 2010 discloses that DNA *polymerases* were *incubated* with *primed DNA templates*

to form a “*ternary* complex,” and then “the ternary complex was immobilized onto the ZMW arrays.” EX1021, 2; EX1018, ¶[0043]; EX2026, 2.

130. The SMRT sequencing workflow requires that the primer:template:polymerase ternary complex be distributed at one complex per ZMW well. If more than one polymerase is incorporating nucleotides in a given ZMW, the multiple separate incorporation events will be read as a single sequence, producing useless data. In fact, Eid 2009 taught that “[a] *single molecule of DNA template-bound Φ 29 DNA polymerase* is immobilized at the bottom of a ZMW.” EX2001, 3 (emphasis added). Neidringhaus also taught “immobilizing *a single functioning DNA polymerase* at the bottom of *each ZMW*.” EX2002, 4 (emphasis added). The PacBio Backgrounder also disclosed that “[a] single DNA polymerase molecule is attached to the bottom of each waveguide” and that “each ZMW provides a window that enables the real-time observation of a single molecule of DNA polymerase as it synthesizes DNA.” EX2021, 4.

131. Knowing this, a POSA would have recognized that the SMRTbell circular template of Travers '075 and Travers 2010 contains a single primer/polymerase binding site to form the required 1:1:1 ternary complex to enable single molecule sequencing. In fact, the “SM” in SMRT sequencing refers to “single molecule.” EX1021, Abstract. Moreover, Travers 2010 teaches that the SMRTbell template “provides *a* site for primer binding” and depicts in Figure 1 a

SMRTbell template including “*a single-stranded region to which a sequencing primer can bind.*” EX1021, Abstract, 3, Fig. 1. Travers ’075 also teaches that SMRTbell templates “*provide the primer and polymerase binding site.*” EX1018, ¶[0067].

132. Implementing a rolling circle replication embodiment eliminates the single molecule capability of SMRT sequencing. A POSA would have recognized that in a rolling circle replication embodiment, the template DNA molecule is not the SMRTbell template, but rather a linear concatemer comprising multiple copies of the SMRTbell template DNA and *multiple copies of the primer/polymerase binding site.*

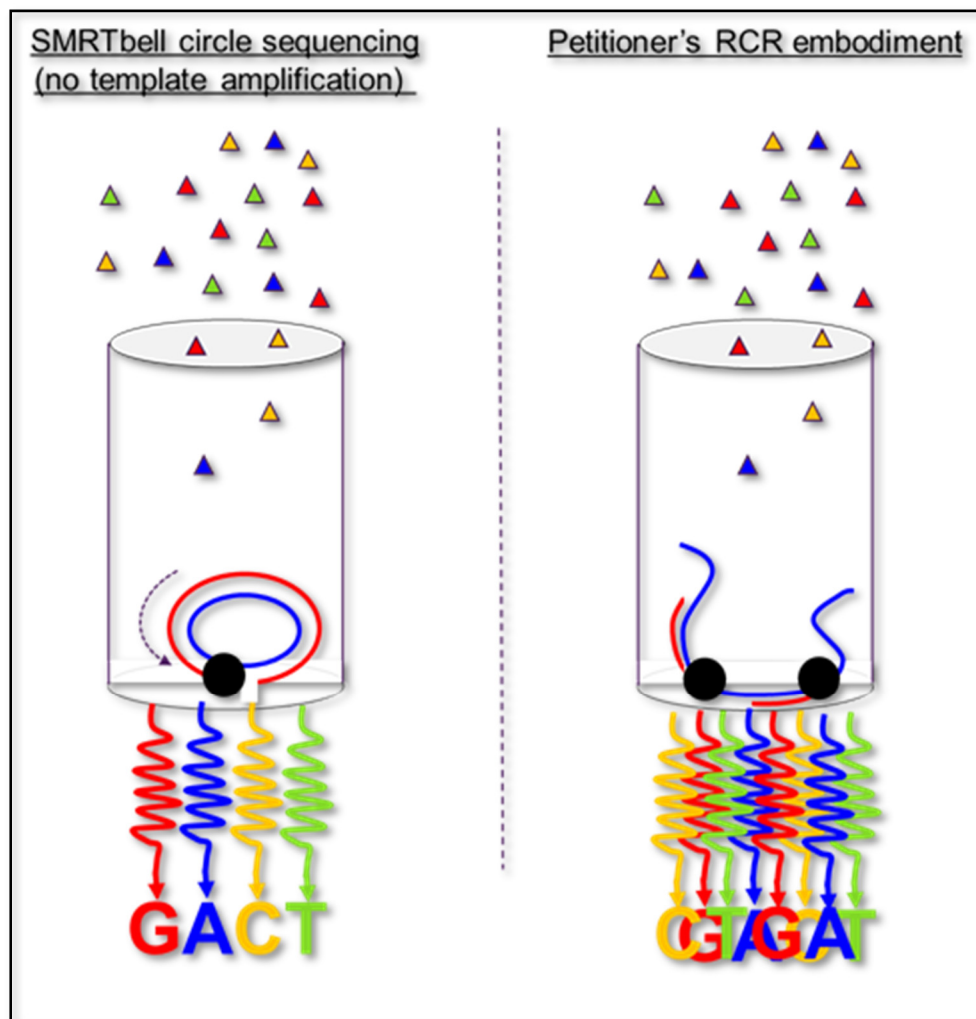
133. A POSA reading Travers 2010 would have expected even the *smallest* rolling circle replication concatemer to have at least two primer/polymerase binding sites. For example, as discussed above, Travers 2010 discloses generating a range of rolling circle replication product sizes, the smallest of which is about 500 nucleotides long. EX1021, Fig. 3. However, Travers 2010 teaches that the SMRTbell circle template has a length of 336 nucleotides, which means that the smallest product at 500 nucleotides represents about 1.5 times around the circle template. EX1021, 4. With 1.5 revolutions around the SMRTbell circle template, or about 3 target copies, there would be at least two copies of the primer/polymerase binding site. EX1021, 4. Thus, a POSA would expect that the

primer and multiple polymerases would bind to the linear concatemer template at multiple sites along the concatemer prior to sequencing. As such, a POSA would have been unable to generate the 1:1:1 ternary complexes required for SMRT sequencing. Thus, with multiple polymerases bound, a POSA would not expect to produce any useable sequence data at all.

134. A POSA would have understood that the ZMW array would not have been able to sequence a multi-primed, multi-polymerase concatemer complex because of the limitations of the SMRT sequencing workflow. In the traditional SMRT sequencing workflow, “[t]he fluorescence signal for each of the analogs is observed by a CCD camera.” EX1021, 4-5; EX1018, ¶¶[0045], [0141]. However, in order to sequence a multi-complex, the SMRT sequencing camera would have to be able to process numerous, concurrent nucleotide flashes occurring from the multiple concurrent polymerase extension reactions taking place in each ZMW. A POSA would have understood that the data from any ZMW containing multiple polymerases engaged in synthesis would be unreadable because a POSA would have no way to resolve the fluorescence signals and identify which of the multiple polymerases in a ZMW was responsible for a given observed signal. Moreover, a POSA would have understood that these concurrent nucleotide flashes would be detected not as amplification of the signal, but rather as readings of different nucleotides occurring simultaneously and therefore the order of the nucleotides

(i.e., the actual sequence) would be impossible to determine.

135. As shown below in the figure, a POSA would have understood that if two or more polymerases generate products in a single ZMW well, the multiple fluorescent pulses from the different polymerases would be indistinguishable and produce unusable data.



136. A POSA would have known that the SMRT sequencing system would register *every* incorporation event detected within an individual ZMW well as part

of the same linear sequence. However, if those signals were coming from different polymerases bound to the same linear concatemer template, a POSA would not be able to decipher the signals to generate any useable sequence data. A POSA would also understand that this problem will only worsen for larger concatemers containing more polymerase binding sites.

137. In sum, a POSA would have understood that this reason alone would have dissuaded a POSA from incorporating Travers '075's rolling circle replication embodiment into SMRT sequencing because a POSA would not have been motivated to modify a functioning workflow and make it unworkable.

3. Problems 2 and 3: A POSA would have known that Travers '075's rolling circle replication embodiment would produce a wide range of concatemer sizes that create a diffusion bias toward smaller templates into the ZMW wells.

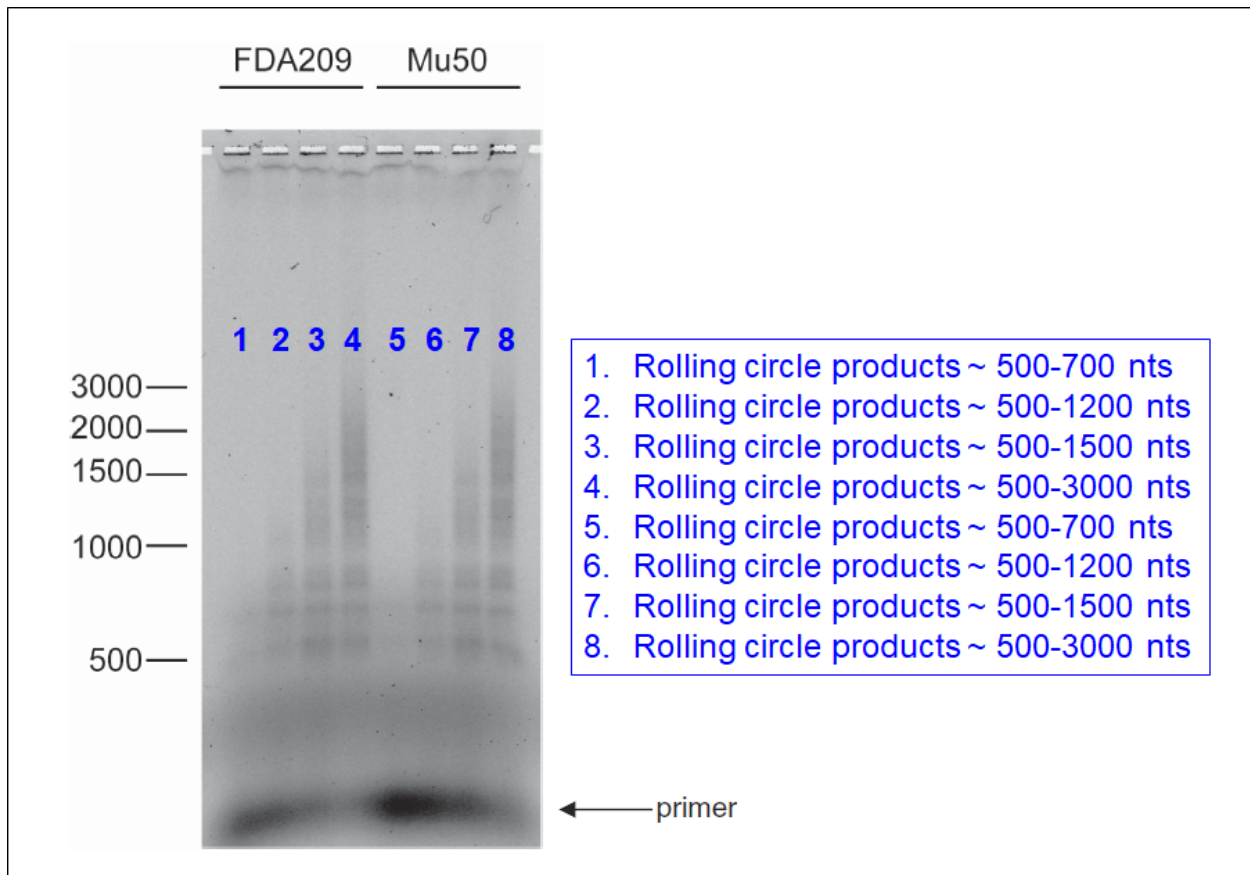
138. A POSA would have also understood that Travers '075's rolling circle replication embodiment would also have been plagued with problems with DNA diffusion into SMRT sequencing's ZMW arrays. As described below, a POSA would have understood that incorporating rolling circle replication in the SMRT sequencing method of Travers '075 and Travers 2010 would generate products with varying sizes. Moreover, a POSA would have understood that SMRT sequencing suffered from a well known limitation when sequencing a mixture of DNA molecules with different product sizes, namely, that smaller DNA products

would be substantially overrepresented in the sequencing data.

139. In Figure 3 shown below, Travers 2010 discloses that performing the rolling circle replication reaction produced a smear of reaction products with a wide range of sizes. EX1021, Fig. 3. Specifically, Figure 3 discloses the bulk extension products of two SMRTbell templates with lanes 1-4 containing the product from a template derived from strain FDA209 at timepoints of 2, 10, 30 and 60 minutes. EX1021, Fig. 3. Lanes 5-8 contain the product from a template derived from another strain, Mu50, at the same timepoints. EX1021, Fig. 3.

140. As can be seen in the approximate sizes of products denoted in the annotated gel in Figure 3, rolling circle replication of both templates generated products 500-700 nucleotides in length at the 2 minute timepoint in lanes 1 and 5. EX1021, Fig. 3. At the 10-minute timepoint in lanes 2 and 6, the products range in size from 500 to 1200 nucleotides. EX1021, Fig. 3. The product size range increases to 500-1500 nucleotides at the 30 minute timepoint, shown in lanes 3 and 7. EX1021, Fig. 3. Eventually, the smaller species continue to grow and accumulate over time, resulting in a smear of products ranging in size from 500 to 3000 nucleotides long at 60 minutes in lanes 4 and 8. EX1021, Fig. 3.

141. As shown in the figure below, the smaller products are present at every timepoint (2, 10, 30, and 60 minutes) even as larger products begin to form and accumulate over time. EX1021, Fig. 3.

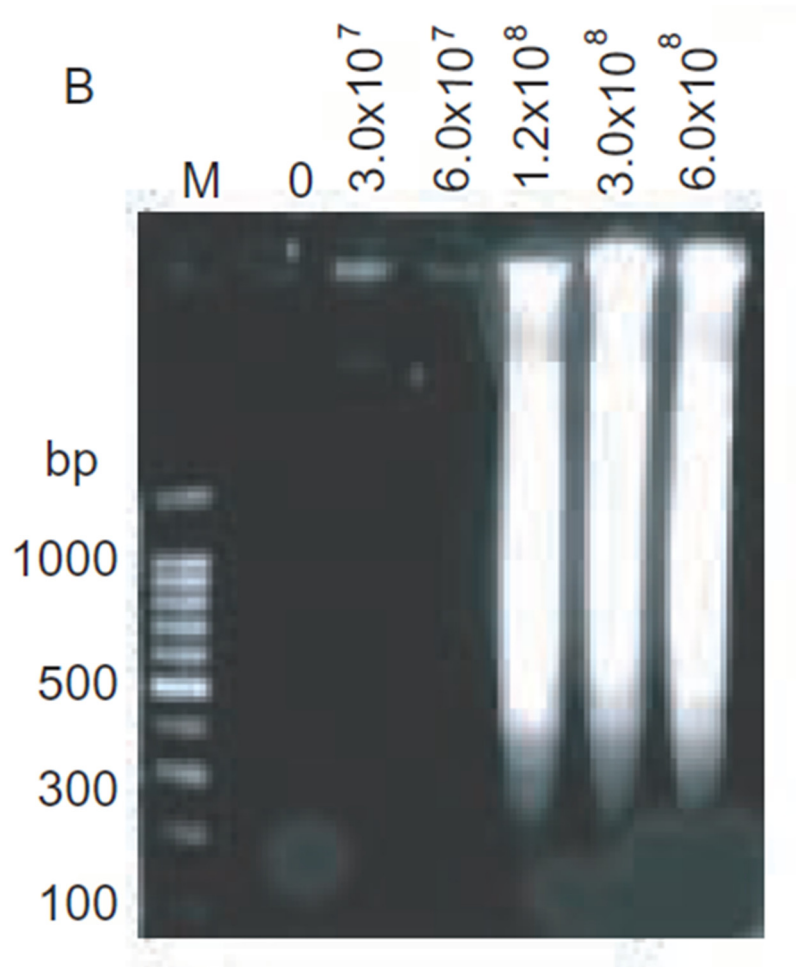


EX1021, Fig. 3 (annotated). A POSA would have understood that rolling circle replication in Travers 2010 initially produces smaller products in the range of 500 nucleotides long before gradually accumulating products ranging in sizes up to 700, 1,200, 1,500, and 3,000 nucleotides in length.

142. Prior to March 20, 2012, the art taught that this smear of products accumulating from rolling circle replication in Travers 2010 was entirely expected. In fact, Demidov 2002 disclosed that products of rolling circle replication “generally exhibit a wide, essentially continuous distribution over length and therefore cannot be resolved by gel electrophoresis *yielding a broad smear of the*

high molecular weight DNAs.” EX2036, 2 (emphasis added). Demidov 2002

similarly depicted this broad smear in Figure 3B below, where lanes 5-7 disclose a broad smear of replication products from different initial inputs of the circular DNA. EX2036, Fig. 3B.



EX2036, Fig. 3B (cropped).

143. Similarly, Liu 1996 discloses a very similar gel showing the increase in length of rolling circle products over a period of 10 minutes to 24 hours.

EX2037, Fig. 6. As shown below, Liu 1996 discloses a broad smear of products

ranging in size from hundreds of nucleotides to over 12,000 nucleotides in length starting at the 3 hour timepoint onwards in lanes 3-6 in the gel in Figure 6.

EX2037, Fig. 6.

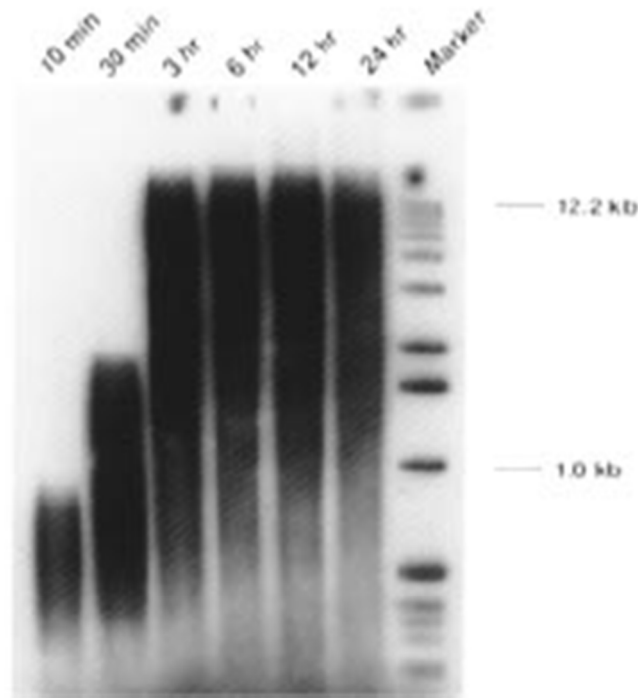


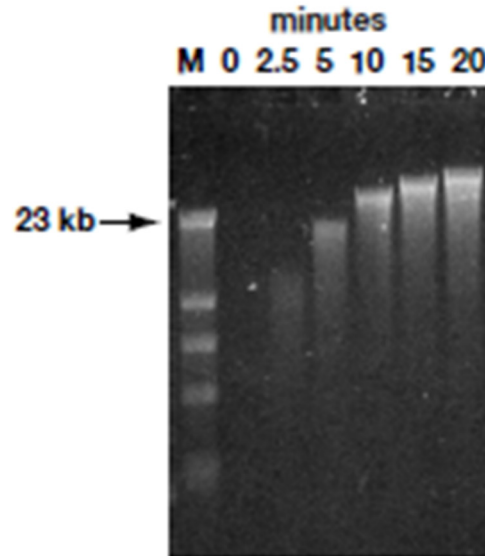
Figure 6. Time course showing the increase in length of rolling circle products. Shown is an autoradiogram from 1% agarose gel analysis of products at several time points in one reaction with 42mer circle 3 as template. Products are radiolabeled by initiating with ³²P-labeled primer.

EX2037, Fig. 6.

144. Another article, Lizardi 1998, discloses a similar broad smear of rolling circle replication products starting even sooner at 2.5 minutes. EX2038, Fig. 3. From these references, a POSA would have understood that rolling circle replication generates products with wide-ranging sizes. Notably, a POSA would have understood that rolling circle replication can generate high molecular weight

products, but that low molecular weight products persist in the reaction. In sum, rolling circle replication generates an enormous diversity of product sizes and there is little control that the researcher has over the product sizes produced.

Fig. 3 Analysis of RCA products. Time course of amplification of circularized probes, catalyzed by ϕ 29 DNA polymerase. Circularized padlock probes were prepared as in Fig. 2b. Aliquots of the RCA reaction were taken at the times indicated, denatured in alkaline buffer and analysed on a 0.7% alkaline agarose gel. The lane marked as 'M' contained phage lambda DNA digested with restriction endonuclease *HindIII*. The largest DNA fragment of 23 kb is indicated by the arrow.



EX2038, Fig. 3.

145. As a result, a POSA would have known that this smear of reaction products from rolling circle replication would create problems if combined with the SMRT sequencing applications of Travers 2010 due to DNA diffusion. Specifically, a POSA would expect the smaller DNA molecules from this smear of products to diffuse much more easily into the ZMW wells. EX2039, Table 1, Fig. 1; EX2040, 4, Fig. 1; EX2041, 4. The art was replete with references teaching that the small diameter of ZMW wells caused a bias towards preferentially sequencing

smaller template molecules because smaller molecules diffused more easily into the ZMW. For example, Grosberg 2010 taught that “when DNA undergoes free diffusion; its diffusion coefficient, which we denote as D , decreases with DNA length N .” EX2041, 4. As a result, a POSA would have known that smaller DNA molecules have a much higher diffusion coefficient than the larger molecules. A POSA would have understood that a diffusion coefficient is a measure of the amount of a substance that can diffuse across a unit area in 1 second. EX2056, 20. If a particular molecule has a higher diffusion coefficient, it will diffuse more efficiently into a particular substance than one with a lower diffusion coefficient.

146. In contrast to the smaller molecules, a POSA would have understood that the larger molecular weight products could not be usefully sequenced using SMRT sequencing methods because of basic principles of DNA diffusion. Thus, a POSA would have understood that in Travers '075's rolling circle replication embodiment, the smaller molecules would drastically outcompete the larger molecules based on well-known diffusion principles.

147. Additionally, in 1996, Smith taught that as the length of DNA grows, the diffusion coefficient of the molecule decreases. As shown in Table 1 below, Smith 1996 taught that shorter bacteriophage DNA molecules had much higher diffusion coefficients (D). EX2039, Table 1. In fact, Smith 1996 taught that as the length of different length restriction fragments of phage DNA increased from 2 to

10.5 μm , the diffusion coefficient decreased from 1.94 to 0.66 $\mu\text{m}^2/\text{s}$. EX2039,

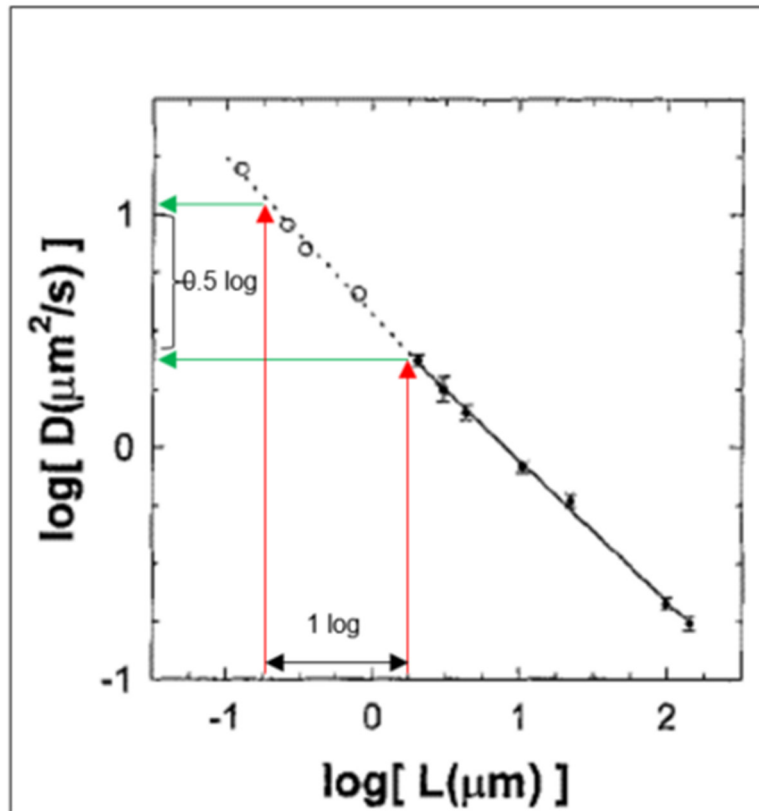
Table 1.

Table 1. Diffusion Coefficients and Inferred Radii of Gyration for TOTO-1 Stained Bacteriophage DNA Molecules				
source	size (bp)	length (μm)	D ($\mu\text{m}^2/\text{s}$)	R_G (μm)
λ concatmer	~309 000	140	0.14 ± 0.01	2.47
λ concatmer	~216 000	98	0.17 ± 0.01	2.03
λ phage	48 502	22	0.47 ± 0.03	0.73
λ <i>Hind</i> III F1	23 130	10.5	0.66 ± 0.04	0.52
λ <i>Hind</i> III F2	9 416	4.3	1.13 ± 0.08	0.31
λ <i>Hind</i> III F3	6 557	3.0	1.43 ± 0.17	0.24
λ <i>Hind</i> III F4	4 361	2.0	1.94 ± 0.10	0.18

EX2039, Table 1.

148. Smith 1996 also generated a log-log plot of the self-diffusion coefficient versus chain length for isolated DNA molecules and found that the “solid line is a linear fit to the data.” EX2039, Figure 1. As shown below, a POSA would have understood that there was a linear relationship between the log of the chain length and the log of the diffusion coefficient of these DNA molecules.

EX2039, Figure 1.

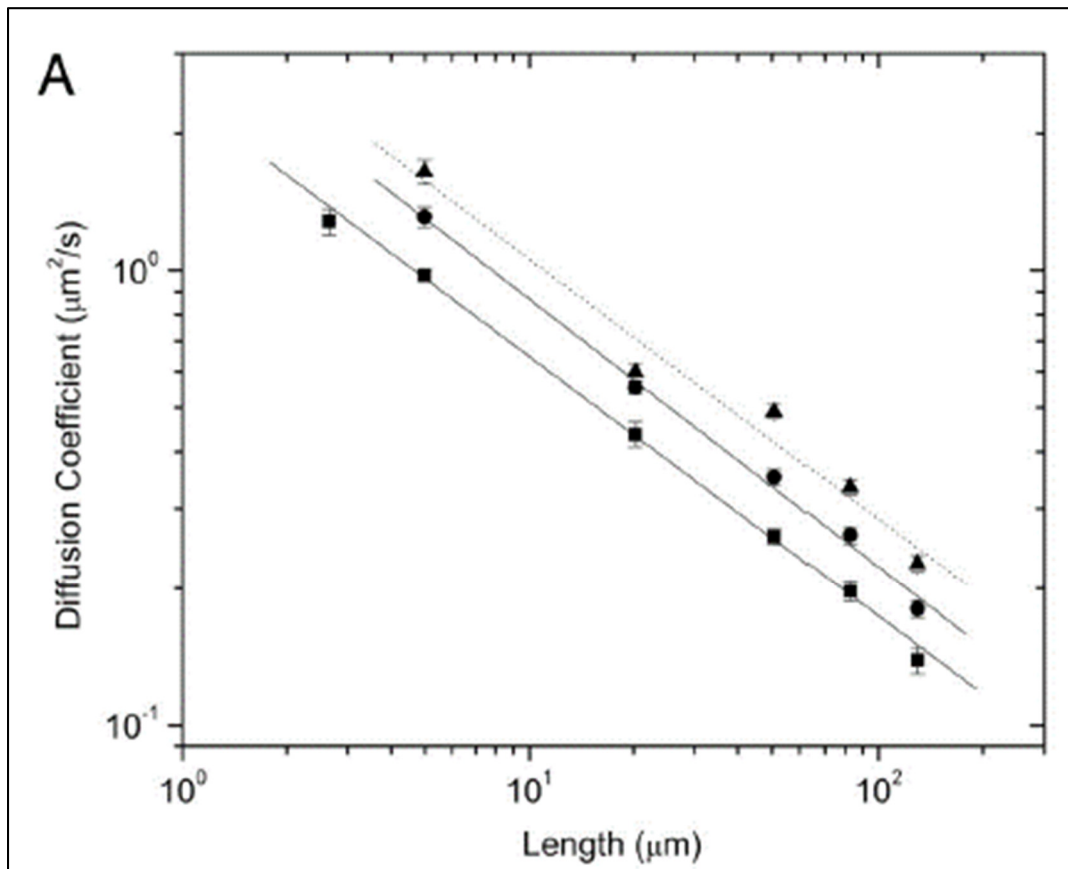


EX2039, Fig. 1 (annotated). From this, a POSA would have understood that longer DNA molecules do not diffuse as easily as shorter DNA molecules. In addition, based on the annotations I have added to the graph, a POSA would have understood that Smith 1996 taught that a 10-fold increase in DNA molecule length “L” (1 log on x-axis) results in approximately a 5-fold decrease in diffusion coefficient “D” (0.5 log on y-axis).

149. Similar to Smith 1996, Robertson 2006 taught that there was a functional relationship between DNA molecule length and the diffusion coefficient. EX2040, 4. In particular, Robertson noted that “power-law scaling was

observed for the relaxed circular, linear, and supercoiled DNA forms.” EX2040, 4.

As shown below, Robertson 2006 disclosed in Figure 1 a very similar graph depicting the linear relationship between diffusion coefficient and chain length of linear (squares), relaxed circular (circles), and untreated supercoiled (triangles) DNA. EX2040, Fig. 1.



EX2040, Fig. 1.

- a) **Problem 2: The well-known diffusion bias for small templates in SMRT sequencing would result in unreliable sequencing data.**

150. A POSA would have understood that as a result of the well-known

inverse relationship between DNA chain length and its diffusion coefficient, smaller template molecules diffused more easily into the small-diameter ZMW wells than larger molecules.

151. The SMRT sequencing workflow utilizes a system called “zero-mode waveguides” in its sequencing workflow. These zero-mode waveguides, also known as ZMWs or ZMW wells, are nanowells fabricated in aluminum on a glass substrate that essentially serve as a visualization chamber to view the activity of a single molecule amongst a background of thousands of labeled nucleotides. EX1021, 4; EX1018, ¶¶[0043], [0141]; EX2027, 4; EX2028, 1; EX2021, 1. Put in simple terms, the ZMWs are used to provide a window for watching DNA polymerase as it performs sequencing by synthesis. EX2021, 1.

152. Travers '075 and Travers 2010 describe using ZMWs in its SMRT™ sequencing methods. Travers 2010 discloses that “[p]olymerization is confined to the bottom of nanostructures known as ZMWs.” EX1021, 4. Travers '075 teaches in Example 1 that the “sequencing reaction was carried out in a zero mode waveguide array having 3000 discrete cores of the ZMWs.” EX1018, ¶¶[0043], [0141], Example 1.

153. One well-known feature of these ZMWs was its small size—ranging from tens of nanometers up to 100 nanometers in diameter. Pacific Biosciences’ own “Technology Backgrounder” taught that “a ZMW is a hole, tens of

nanometers in diameter....” EX2021, 1, 3. Schadt 2010 confirmed this by teaching that ZMWs were “tens of nanometers in diameter fabricated in a 100 nm metal film deposited on a glass substrate.” EX2023, 5. In addition, other references such as Levene 2003 depicted “a zero-mode waveguide 50 nm in diameter and 100 nm long.” EX2029, 2, Fig. 2. Likewise, Korlach 2008 taught experimenting with a wide range of ZMW diameters ranging in size from 70-100 nm. EX2030, 4. Korlach also disclosed that “[i]ncreased DNA polymerase loading was observed with larger ZMW diameters” and that “[s]ingle active polymerase loading to ~30% yield was achieved over a wide range of ZMW diameters (70–100 nm).” EX2030, 4. McCarthy 2010 also confirmed that ZMWs ranged in size from “70 nm in diameter by 100 nm in depth.” EX2031, 1.

154. Similarly, Gupta 2008 taught using “SMRT chips, each made up of a 100-nm-thick metal film and containing thousands of zero-mode waveguides (ZMWs; see Glossary), which are *cavities of 10–50 nm in diameter.*” EX2027, 5 (emphasis added). Eid 2009 also disclosed a “ZMW 100 nm in diameter.” EX2001, 134.

155. A POSA also would have understood that in the SMRT sequencing workflow, single molecule circular SMRTbell templates combined with a single polymerase enzyme are then added to the ZMW array. EX1021, 2; EX2001, 134. Then, the complexes diffuse through the aqueous medium into the ZMW wells,

where they are immobilized on the bottom of the well for sequencing with only one complex per ZMW well. EX1021, 2; EX2001, 133; EX1018, ¶¶[0140]-[0149]. As such, only ZMW wells containing a single polymerase and a single template would be expected to produce any potentially useable sequence data because a POSA would understand that ZMW wells containing no polymerase or no template produce no sequence data. Similarly, ZMW wells with two or more polymerases and templates produce no useable sequence data.

156. Before March 20, 2012, a POSA also would have understood that according to Poisson distribution³ statistics, only about 30% of the ZMW wells will contain single molecules of polymerase and DNA template for sequencing. Specifically, Eid 2009 taught that the Poisson distribution showed that approximately 30% of the ZMWs are occupied with a single polymerase and single template molecule for sequencing. Indeed, Eid disclosed that “polymerase molecules are randomly distributed among the ZMWs, leading to a Poisson distribution of occupancy. At optimal loading, the distribution is 36.8% empty ZMWs, 36.8% *with just one polymerase*, and 26.4% with two or more.” EX2001,

³ A POSA would have understood that following a Poisson distribution means that the ZMW loading process happens independently and randomly over time and the mean rate of occurrence is constant over time. EX2067, 11-12

4. Korlach 2008 also taught that “[s]ingle active polymerase loading to ~30% yield was achieved over a wide range of ZMW diameters (70–100 nm).” EX2030, 4. Thus, a POSA reviewing these two references would have expected to see only about 30% of the ZMW array with single-molecule occupancy. As such, only 30% of the ZMW would be expected to produce any potentially useable sequence data because a POSA would understand that ZMW wells containing no polymerase or no template produce no sequence data. Similarly, ZMW wells with two or more polymerases and templates produce no useable sequence data. EX2001, 4.

157. In the case of Travers ’075’s rolling circle replication embodiment, a POSA would have understood that the majority of that 30% occupancy would be filled by the smaller templates rather than the larger templates due to basic diffusion principles. As a result, a POSA would have expected these smaller templates to not provide useful sequencing data because they lack sufficient sequencing redundancy.

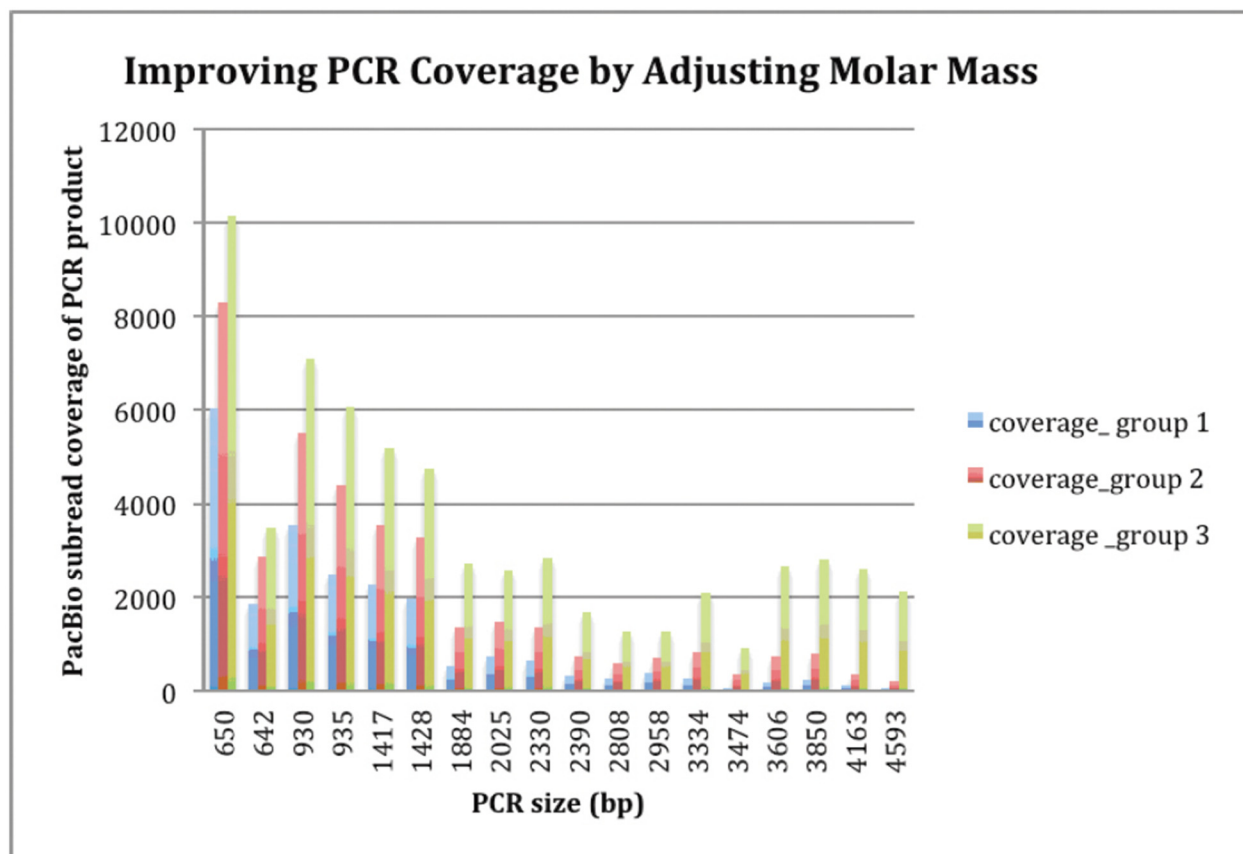
158. Schadt disclosed in 2010 that “the DNA polymerase and DNA template to be sequenced are delivered to ZMWs via a *random diffusion process*.” EX2023, 5. In view of the known inverse relationship between a DNA molecule’s length and its diffusion coefficient, a POSA would have expected that the random diffusion process for loading ZMWs would have resulted in a race between the vast range of different-sized rolling circle replication products. In addition, a

POSA would have understood that the much smaller products would generally win this race due to their higher diffusion coefficients. As a result, the resulting sequencing data would predominately feature small template molecules, which as discussed above, produce poor, unreliable data because these small templates lack sufficient concatemer copies to conduct redundant sequencing needed to overcome the very high error rate of the PacBio system.

159. Moreover, the contemporaneous literature shows size bias was a significant issue for PacBio sequencing. Indeed, Zhang 2012 taught that “there is a loading bias against large DNA fragments in the PacBio sequencing process” and that “[s]maller PCR products will load into the PacBio wells with a greater efficiency than larger PCR products.” EX2028, 61. Zhang 2012 also taught that “[w]hen PCR products ranging from 500 bp to 5 kb are pooled and sequenced together using PacBio, the smaller products have a substantially higher coverage than the larger products resulting in poor quality or incomplete sequences for the larger PCR products.” EX2028, 61. In addition, Zhang 2012 set out to test the effect of adjusting the molar ratio of the PCR products when pooling them together based on the PCR size and concentration. EX2028, 61. In doing so, Zhang 2012 tested three groups: “group one was our control, where we loaded equal DNA mass for each PCR product; for group two, we pooled each PCR product at the equal molar mass; for group three, we adjusted the molar mass ratio based on the size of

the PCR product, increasing the molar mass with size.” EX2028, 61.

160. As shown in Figure 1, Zhang 2012 reported that Group 1 “resulted in much higher coverage for the smaller PCR products while the longer PCR products were barely covered.” EX2028, 61. For example, the smallest PCR product (642 bp) had a coverage of 2000, while the PCR product nearly five times larger at 2958 bp had a coverage nearly ten times smaller. EX2028, Fig. 1. After pooling each PCR product at equal molar masses in Group 2, there was still a nearly six-fold difference in coverage between the smallest product and the 2958 bp product. EX2028, Fig. 1. In Group 3, where the molar mass ratios were increased with size, there was still a three-fold difference in PCR coverage between the smallest 642 bp product and the 2958 bp product. EX2028, Fig. 1. Thus, even after all of these adjustments, smaller products still had substantially higher coverage than larger products.

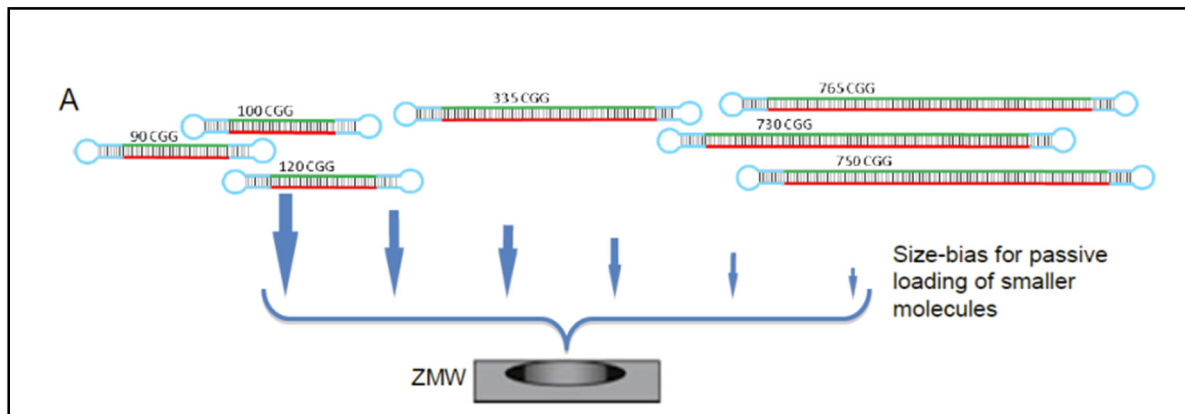


EX2028, Fig. 1.

161. Coupland 2012 similarly disclosed that “[t]here is a loading inefficiency of larger molecules because of their lower diffusion coefficient, as well as the disparity between the molecule’s hydrodynamic radius and the very small zero-mode waveguide (ZMW).” EX2042, 4. Coupland specifically noted the size of the ZMW wells as one reason why there was a loading inefficiency for larger molecules in SMRT sequencing. EX2042, 4. Quail 2012 also taught that “[c]urrent PacBio protocols favor the preferential loading of smaller constructs, resulting in average subread lengths that are significantly shorter than the often

quoted average read lengths.” EX2005, 7.

162. For example, Loomis 2012 taught that the “[p]assive loading of the SMRT cell results in a *selection bias for the loading of smaller alleles* into the ZMWs and, consequently, a length bias of the counts of alleles.” EX2046, 6 (emphasis added). In addition, Loomis 2012 disclosed a diagram illustrating the “bias for passive diffusion loading of smaller molecules into zero mode waveguides (ZMWs) of a SMRT Cell prior to sequencing.” EX2046, Supp. Fig. 4A.



EX2046, Supp. Fig. 4A.

163. Other later references confirm this observation regarding the size bias in PacBio sequencing: “one known bias of the PacBio platform is the preferential loading of shorter sequences into zero-mode waveguides (ZMWs, essentially ‘wells’), thus biasing the resulting community toward members having shorter sequences.” EX2022, 4. Even in 2017, Larkin explained, “[a] critical *limiting step*

of SMRT sequencing is the loading of long DNA templates into ZMW confinements.” EX2057, 2. Larkin also taught that “[m]ismatch between the equilibrium hydrodynamic diameter of long DNAs ... and the ZMW diameter ... creates an *entropic barrier to molecular entry under diffusive conditions*. Under diffusive conditions this barrier *biases entry of short DNA templates over long ones*.” EX2057, 2. Thus, a POSA would have understood that in the milieu of rolling circle replication products, ZMWs favored sequencing smaller template molecules as opposed to larger DNA molecules because of the well-known size constraints of the ZMW wells as well as basic diffusion principles. However, sequencing just the smaller template molecules would not provide enough target DNA copies to be able to resequence the target DNA sufficiently and achieve a desirable higher EQV as taught in Travers 2010. EX1021, Fig. 7. As discussed above, this problem would not occur when using similarly sized circular templates for sequencing as disclosed in Travers 2010.

164. In addition, a POSA would have understood that sequencing the shorter, truncated rolling circle replication products would also not meet the elements of claims 1-21. In particular, claim 1 requires “*confirming the presence of at least one sequence read derived from each of the original first and second strands of the adapter-DNA molecules*.” EX1001, claim 1 (emphasis added). Using these shorter, truncated products, a POSA would not be able to confirm the

presence of a sequence read from *each* strand, as recited in claim 1, because they generally would not contain a complete copy of each strand. As a result, a POSA would understand that these smaller products do not meet the elements of claim 1. Moreover, if a concatemer template *was* large enough to permit confirming the presence of a top strand and a bottom strand, it would have multiple primer/polymerase binding sites and therefore fall victim to Problem 1, above.

b) Problem 3: Larger concatemer products would be outcompeted by smaller products for diffusion into the ZMW wells for SMRT sequencing.

165. In view of the extensive teachings in the art, a POSA would have known that Travers '075's rolling circle replication embodiment, which produces a smear of DNA products with varying sizes, was incompatible with SMRT sequencing due to commonly understood DNA diffusion principles. As mentioned above in detail, the art taught that DNA diffusion imposed a bias for sequencing shorter templates over larger molecules. As a result, a POSA would have known that the small diameter of ZMW wells would have preferentially favored sequencing smaller template molecules due to the higher diffusion coefficient expected for smaller molecules.

166. However, larger molecules would not fare any better. Larger concatemers generated from rolling circle replication would instead be outcompeted for occupancy of the ZMW wells by the smaller products due to

DNA diffusion bias. EX2041, 3; EX2039, Fig. 1; EX2040, Fig. 1. Dr.

Quackenbush has also not accounted for the well-known diffusion issues affecting large concatemer products that are associated with the SMRT™ sequencing applications of Travers 2010. A POSA would have also known that the large concatemer molecules produced from rolling circle replication would not readily diffuse into the narrow ZMW wells and would be inefficient templates for SMRT™ sequencing.

167. Notably, if a POSA were to follow the normal SMRT sequencing workflow disclosed in Travers '075 and Travers 2010 that uses single molecule circle sequencing *instead of including an additional rolling circle replication step*, the issues of diffusion bias and target DNA copy number would no longer apply. Therefore, a POSA would have had no reason to combine Travers '075's rolling circle replication embodiment with the SMRT sequencing applications of Travers 2010.

168. Moreover, I understand from reviewing Dr. Quackenbush's deposition transcript that he did not consider these diffusion issues or target DNA copy number when preparing his declaration. As his testimony reflects, Dr. Quackenbush did not consider how the design of the ZMW wells preferentially favor sequencing smaller molecules rather than larger molecules because of DNA diffusion issues. In fact, Dr. Quackenbush repeatedly testified that he did not

consider these issues:

Citation	Dr. Quackenbush's Testimony
15:24-25	"I don't believe I talk about diffusion coefficients in my declaration...."
18:6-7	"Again, I don't believe I discussed DNA diffusion coefficients in the context of my report...."
18:16-17	"As I told you earlier, I don't believe I discussed diffusion coefficients in my report...."
23:8-9	"To the best of my recollection, I do not use the term 'diffusion coefficient' anywhere in my report...."
24:1-2	"I don't recall anywhere in my declaration where I discuss diffusion coefficients..."
30:12-13	"I do not believe diffusion of DNA is discussed in my declaration."
31:12-18	"the phrase 'diffusion of DNA' does not ... appear anywhere in my declaration."

EX2045, 15:24-25, 18:6-7; 18:16-17; 23:8-9; 24:1-2; 30:12-13; 31:12-18.

169. Thus, a POSA would have understood that combining Travers '075's rolling circle replication embodiment with the SMRT sequencing applications of Travers 2010 would produce a wide range of products incompatible with SMRT sequencing because of the well-known DNA diffusion bias for smaller DNA products and the fact that smaller products would not provide a sufficient number of copies to achieve a usable sequencing readout.

4. Problem 4: A POSA would have known that Travers '075's rolling circle replication embodiment would produce structures that would not physically fit within the SMRT sequencing ZMW wells.

170. As of March 20, 2012, a POSA would have been aware that the

ZMWs used in SMRT sequencing have a very narrow diameter. EX2029, Fig. 2A; EX2027, 5; EX2030, 4; EX2001, 134; EX2021, 1; EX2023, 5; EX2031, 1.

However, a POSA would have understood that the small diameter of the ZMWs was important for reducing background fluorescence of other nucleotides in solution during sequencing. It was known that other single molecule detection techniques utilize low nanomolar concentrations of labeled nucleotides during sequencing because at higher concentrations, the detection volumes of these microscope systems are flooded with hundreds or thousands of labeled molecules, which creates a high background noise level that makes it nearly impossible to detect individual fluorophores for sequencing by synthesis. EX2021, 2-3.

However, the problem with limiting the concentration of labeled nucleotides in solution is that DNA polymerase requires a high concentration of labeled nucleotides, without which the speed, accuracy and processivity of the enzyme suffers. EX2021, 2-3.

171. The SMRT™ sequencing platform disclosed in Travers '075 and Travers 2010 addressed this problem by performing sequencing using the SMRT cell, which contains thousands of ZMWs with very small detection volumes. EX2021, 2-3. By utilizing ZMWs with very small volumes, a single incorporation event can be observed against the background created by the high concentration of fluorescently labeled nucleotides. EX2021, 2-3. This makes possible real-time

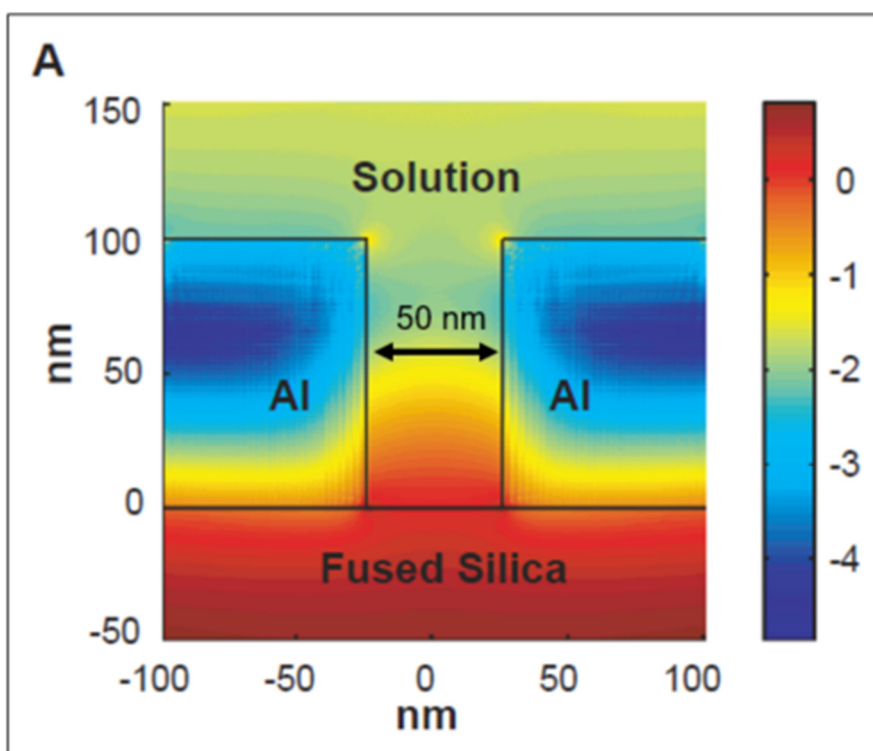
observation of a single molecule of DNA polymerase as it synthesizes DNA.

EX2021, 2-3.

172. The art taught that the small size of the ZMWs played an important role in preventing laser light from passing through the entire ZMW well and exciting unincorporated fluorescently tagged nucleotides. In fact, it was well-known that “[w]hen the ZMW is illuminated through the transparent substrate by laser light, the wavelength of the light is too large to pass through the waveguide’s aperture.” EX2021, 3. In addition, Schadt 2010 disclosed that “[t]he small size of the ZMW prevents visible laser light, which has a wavelength of ~600 nm, from passing entirely through the ZMW. Rather than passing through, the light exponentially decays as it enters the ZMW. Therefore, by shining laser illumination up through the glass into the ZMW, only the bottom 30 nm of the ZMW becomes illuminated.” EX2023, 5.

173. Eid 2009 explained that the narrow diameter of ZMWs “provides excitation confinement in the zeptoliter (10^{-21} liter) regime, enabling detection of individual phospholinked nucleotide substrates against the bulk solution background as they are incorporated into the DNA strand by the polymerase.” EX2001, 134.

174. As shown in the annotated figure from Levene depicting laser light excitation in a ZMW, the opening is only 50 nm in diameter.



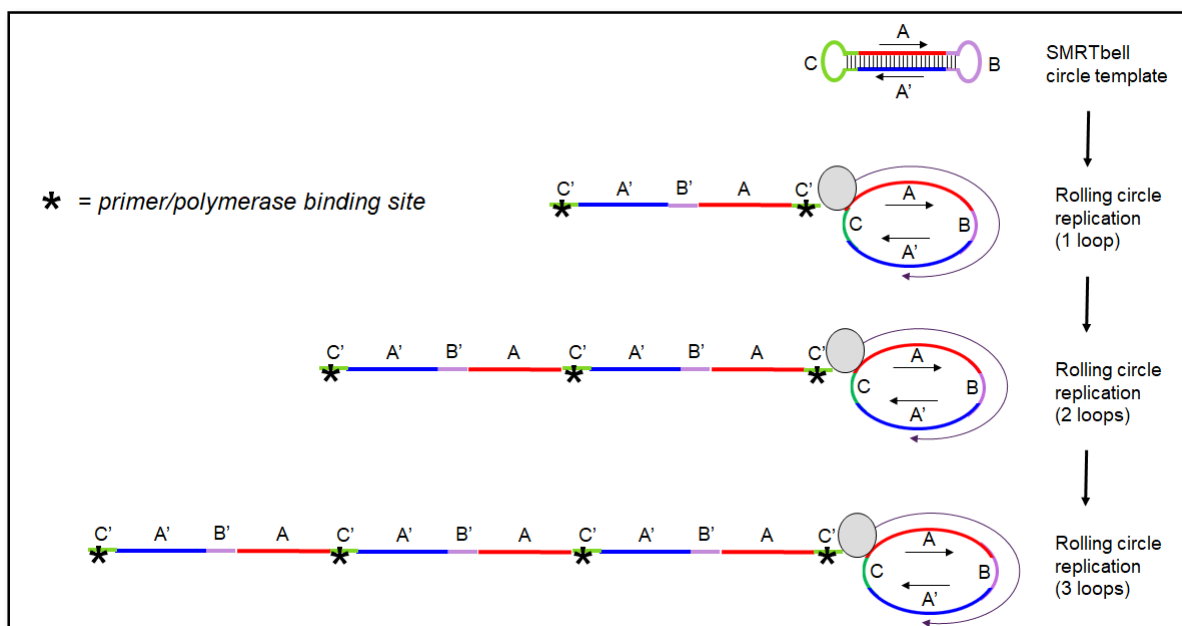
EX2029, Fig. 2A (annotated); EX2001, Fig. 1.

175. As discussed above, in the SMRT sequencing workflow, a single molecule circular SMRTbell template and the polymerase enzyme diffuse through the ZMW wells where they are immobilized on the bottom of the well for sequencing. EX1021, 2; EX2001, 134; EX1018, ¶¶[0140]-[0149].

176. Thus, a POSA would have understood that the size of the ZMWs prevent the excitation laser light from traveling through the entire well and exciting the unincorporated fluorescently tagged nucleotides. As a result, the art taught that the small ZMW wells were essential to reducing background fluorescence in the SMRT™ sequencing results.

177. Knowing this, a POSA would have understood that there was an additional problem with Travers '075's rolling circle replication embodiment that was overlooked by Dr. Quackenbush. A POSA would have expected the narrow ZMWs to present physical constraints on the larger concatemer products of Travers '075's rolling circle replication embodiment and impede their entry into the ZMW.

178. A POSA would understand that these ZMWs present physical or geometrical constraints on large concatemer products. As discussed above and shown in the figure below, a POSA would have known that linear concatemers produced from rolling circle replication would contain many repeating stretches of complementary DNA sequences. Dr. Quackenbush agrees that a POSA "would have understood that the concatemer molecules would have the same sequence (e.g., barcode and target molecule)." EX1002, ¶114. As shown below, I have depicted in the figure the repeating complementary stretches of DNA as "A" and "A'" produced from a rolling circle in a linear fashion.



179. As discussed above, a POSA knowing that SMRT sequencing has an error rate of 10-20% would have desired to sequence as many copies of the template DNA as possible to achieve higher sequencing redundancy and thus a higher EQV for more reliable sequencing data. EX1021, 6; EX2006, 177; Section XI.C.1. Normally, this redundancy can be achieved by utilizing SMRT sequencing of the circular templates of Travers '075 and Travers 2010, where the *same circular SMRTbell template* is sequenced over and over again. EX1021, 6; EX2006, 177. However, in Travers '075's rolling circle replication embodiment, this redundancy can only be achieved by sequencing a long, linear concatemer molecule comprising many repeating sequences of complementary DNA. EX1021, 6; EX2006, 177. A POSA would have understood that these repeating complementary stretches of DNA would hybridize to form complex secondary and

tertiary structures that would have difficulties physically fitting into the narrow ZMW wells for SMRT sequencing.

180. In order to demonstrate that repeating concatemer copies of DNA will form complex secondary and tertiary structures, I performed in-silico DNA structure modeling with the same template sequences disclosed in Travers 2010. I followed the methods disclosed in Travers 2010 to perform my in-silico modeling, starting with Travers 2010's "short" (*S. aureus* fragment) template and amplifying it with the disclosed primers for each respective template. EX1021, 2.

181. First, I located the sequence of the methicillin-sensitive strain of *S. aureus* FDA 209 from the American Type Culture Collection⁴. Then, using the PCR primers disclosed in Travers 2010, I modeled the PCR reaction ("in-silico PCR") using FastPCR⁵, which generates an expected amplicon based on the input primers and template sequence. The resulting amplicon is shown below:

Primers:

5' - GTACGGGTCTCACCCGGTTAACTGCACCTGCATTAA - 3' and
5' - CCTAAGGTCTCGGAAGGAAATTATTTGAAAAAGA - 3'

⁴ The genomic DNA for *S. aureus* FDA 209 can be found at the following URL: <https://www.atcc.org/products/6538d-5>.

⁵ FastPCR is a commonly used tool in the art to perform in silico PCR and has been available for use prior to March 20, 2012. EX2058; EX2059.

In-silico PCR output based on FastPCR
(<https://primerdigital.com/fastpcr.html>)
Primer handles denoted by blue or red color, homologous sequence
w/template is underlined

In-silico PCR output from FastPCR⁶: (total of 149bp)

```
5'
CCTAAGGTCTCGGAAGGAAATTATTTGAAAAAAGAATTAGATGGCTTTAATATCACAAATTCCTCATAAAGAACGTATCATACCGT
ATTTAGATCATGTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACCGGGTGAGACCCGTAC 3'
3'
GGATTCCAGAGCCTTCCTTTAATAAAGCTTTTTTCTTAATCTACCGAAATTATAGTGTTAAGGAGTATTTCTTGCATAGTATGGCA
TAAATCTAGTACAACCTACTTGTTCGCTAATTACGTCCACGTCAATTGCGCCACTCTGGGCATG 5'
```

182. Since the primers contain a restriction site for the enzyme BsaI, I modeled the restriction digest of the short and long templates using molbiotools's Restriction Analyzer⁷, a commonly used tool in the field as of March 20, 2012. The in-silico digest output is shown below:

```
5'      GAAGGAAATTTATTTGAAAAAAGAATTAGATGGCTTTAATATCACAAATTCCTCATAAAGAAGCGTATCATACCGTATTTAGATCATGTTGATGAACAAGCGATTAAATGCAGGTGCAGTTAAC      3'
3'      CTTTAATAAAGCTTTTTTCTTAATCTACCGAAATTATAGTGTTAAGGAGTATTTCTTGCATAGTATGGCATAAATCTAGTACAACCTACTTGTTCGCTAATTACGTCCACGTCAATTGGCCC      5'
```

183. Afterwards, the digested PCR products were ligated to the hairpin-forming oligonucleotides disclosed in Travers 2010. EX1021, 2. The sequence of

⁶ I generated the blue and the red annotations indicating the primer handles. FastPCR only outputs the amplicon.

⁷ Restriction Analyzer was used to simulate a restriction reaction and check if the enzymes identified were able to cut the fragment at the expected sites. This tool can be found at the following URL: <https://molbiotools.com/restrictionanalyzer.php>. A similar tool, NEBcutter, was available prior to March 20, 2012. EX2060.

the circular SMRTbell template after hairpin ligation is shown below:

Hairpin adapters

5' - CTTCTCTCTCTCTTTTCCTCCTCCTCCGAAGAAGAAGCCGAGAGAGA - 3'

5' - CGGGTTTGTGTGCAAAGCCTAAACCAATATTGATACATTAGCAACAAA - 3' .

Circular product after hairpin ligation:

184. Next, I performed in-silico rolling circle replication of varying lengths, from three full loops (6× copies), five full loops (10x copies), and ten full loops (20× copies) around the SMRTbell templates⁸. EX1021, 4-5, Fig. 3. In order to visualize the DNA structures of these rolling circle replication products, I used the secondary structure prediction tool from Dr. David Mathews' Lab at the University of Rochester Medical Center⁹ to generate the three lowest free energy

⁸ I was unable to generate the generate the secondary structures of concatemer sequences expected from rolling circle replication around the template of the long phage genome fragment in Travers 2010 because of the size limitations of the secondary structure tool.

⁹ This secondary structure tool can be found at the following URL: <http://rna.urmc.rochester.edu/RNAstructureWeb/>. This secondary structure tool has

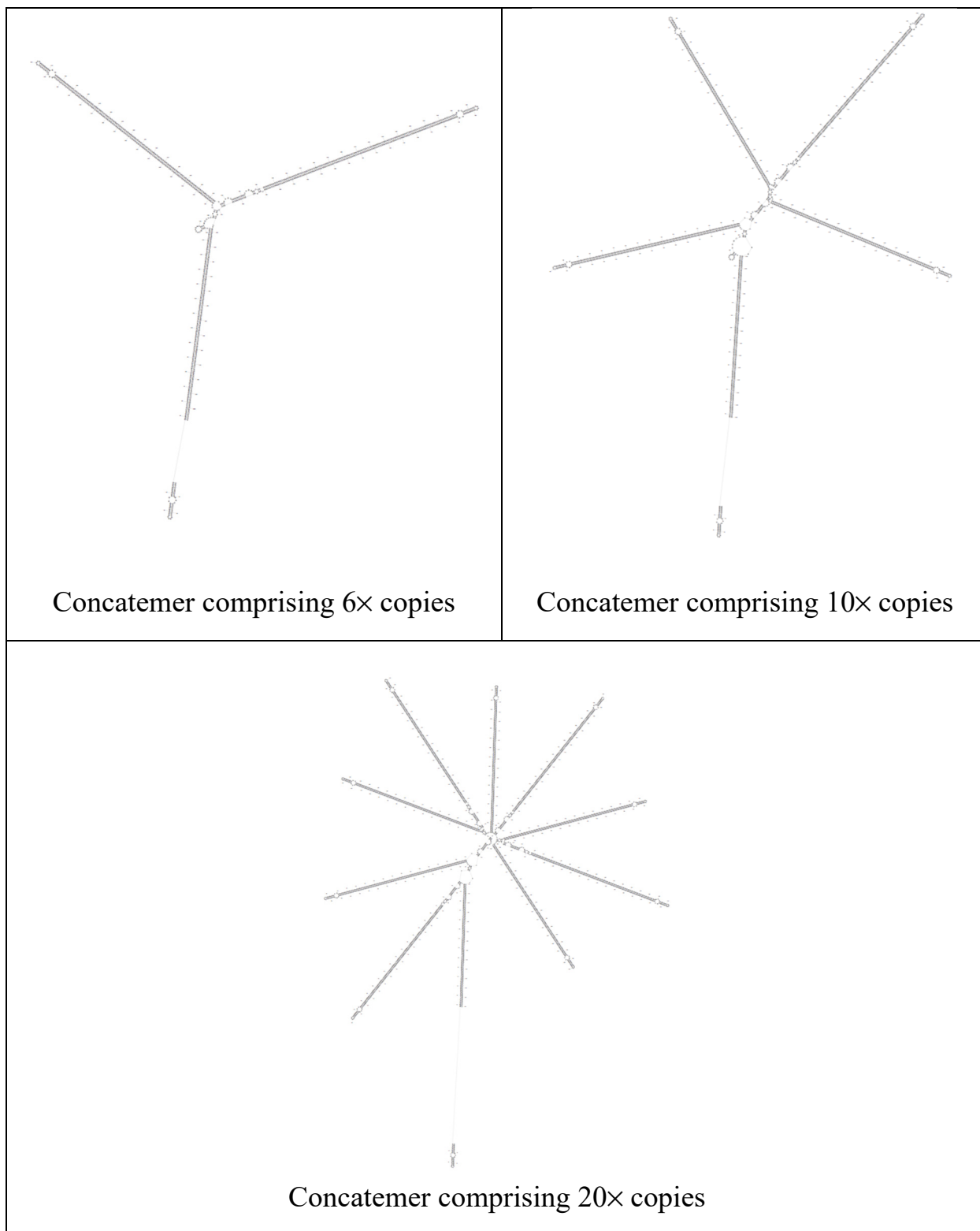
structures for each rolling circle replication product at room temperature (22°C/295.15K)¹⁰. EX2069; EX2070; EX2071.

185. As shown below, the numerous repeating stretches of complementary DNA would have been expected to hybridize with each other and form a variety of complex, star-shaped structures. EX2069; EX2070; EX2071. The predicted structures of the 6×, 10×, and 20× concatemers are shown below.

Expected concatemers from Travers '075's rolling circle replication embodiment using the <i>S. aureus</i> FDA 209 template in Travers 2010

been available prior to March 20, 2012. EX2061.

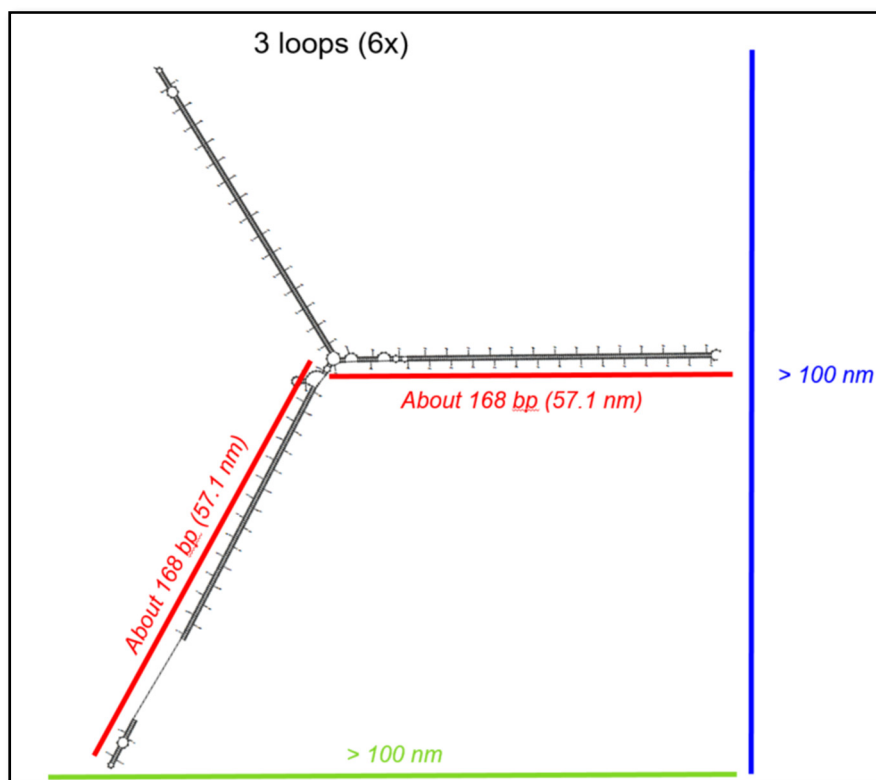
¹⁰ I used the secondary structure prediction tool to generate these rolling circle replication products at room temperature because PacBio sequencing occurs at room temperature. EX2026, Supp. 2.



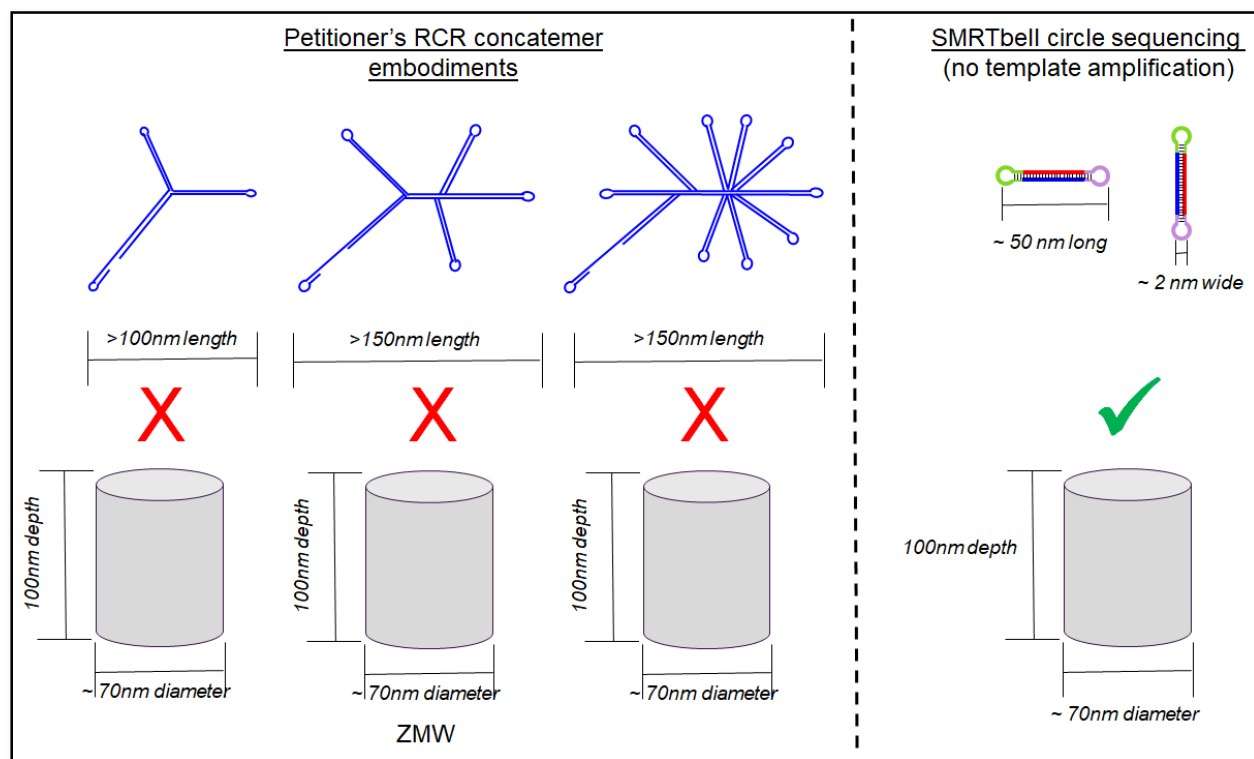
186. In addition, a POSA would have understood that these large, star-

shaped concatemer molecules would be well over 100nm in size and would not readily fit into the narrow ZMWs, which were understood to be ≤ 70 nm in diameter. *See* Section XI.C.3.a.

187. As shown below in the figure, I calculated the approximate length and width of the secondary structure of the concatemer formed after 3 full loops (6x copies) around the SMRTbell template. Given that DNA has a length of approximately 0.34 nm, I calculated that each arm of the star-shaped structure (which is approximately 168bp), would be approximately 57 nm. While it is challenging to calculate the exact width and height of the folded concatemer, particularly as the exact orientation of each arm will vary slightly in solution, both the width and height of this structure encompass two arms. Therefore, I calculate that both the width and height of the structure would exceed 100 nm.



188. While the depicted star-shaped secondary structures above are only two-dimensional, a POSA would understand that the precise three-dimensional structure of the folded concatemer molecule would also fluctuate in solution. Therefore, any individual dimension could span at least two arms of the star-shaped structure. A POSA would therefore not have expected these concatemer molecules to readily fit in the ZMW wells even when considering the tertiary structures of these molecules. As shown in the schematic below that depicts the large concatemers shown to scale with the size of the ZMWs, the concatemers would have difficulties physically fitting within the narrow wells of Travers '075 and Travers 2010.



189. The left panel of the figure above depicts the large concatemers of Travers '075's rolling circle replication embodiment having difficulties fitting into the ZMWs with a diameter of 50-70 nm and 100 nm in depth. EX2029, Fig. 2; EX2030, 4; EX2027, 5; EX2001, 134; EX2021, 1; EX2023, 5; EX2031, 1. As an example, I have used the structures formed from the concatemer after 3 full loops (6x). In the right panel, I have included the width and length of the single molecule SMRTbell templates of Travers 2010, which are not amplified prior to sequencing. As can be seen in the figure above, a POSA would have expected such a single-molecule SMRTbell template to easily fit into the ZMWs. Since the width of a DNA duplex is approximately 2 nm in diameter and the length of the SMRTbell

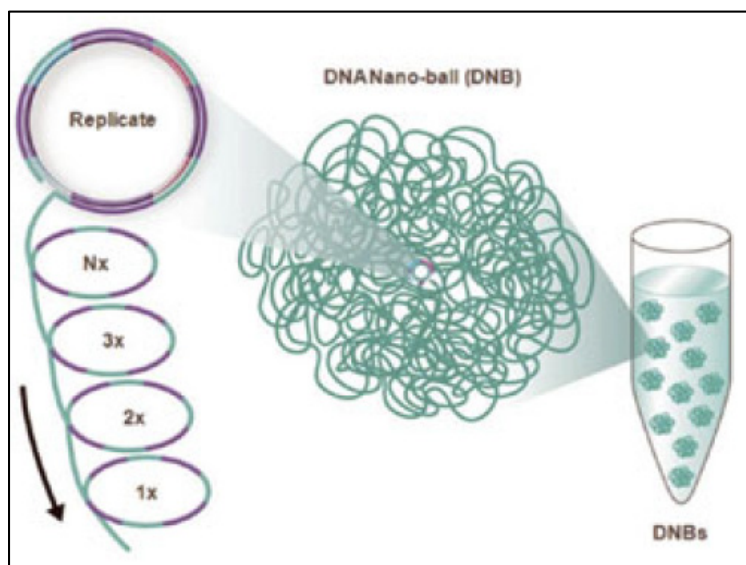
template no longer than 50 nm¹¹, it should easily fit within the small ZMW wells.

EX2063, 2. Even Travers 2010 expressly discloses that the SMRTbell circle templates were designed with the geometry of the ZMW wells in mind. EX1021, 3. In fact, Travers 2010 stated that in “deciding on a format for SMRT™ sequencing, a number of factors were considered, including....*compatibility with ZMW geometry.*” EX1021, 3 (emphasis added).

190. Moreover, a POSA would have also understood that the prior art sequencing workflow of Complete Genomics further supports my opinion that utilizing a rolling circle replication step produces large complex structures. Complete Genomics utilized a similar rolling circle replication step to Travers '075's rolling circle replication embodiment. EX2047, 1-2; EX2004, 1068; EX2002, 4331, 4333. Specifically, in Complete Genomics' system, circular template molecules were copied using a rolling circle replication to produce long concatemers of repeating copies of template DNA. EX2047, 1-2; EX2004, 1068; EX2002, 4331, 4333. Drmanac 2010 referred to these large, complex DNA molecules as “DNA nanoballs.” EX2047, 1. As shown below in the figure, Hui

¹¹ Assuming that the length of the SMRTbell template is 168 bp long and knowing that each nucleotide is 0.34 nm long, the approximate length of the template is around 50 nm. EX2063, 2.

2012 taught that these DNA nanoballs, formed from rolling circle replication, were extremely large and complex structures.

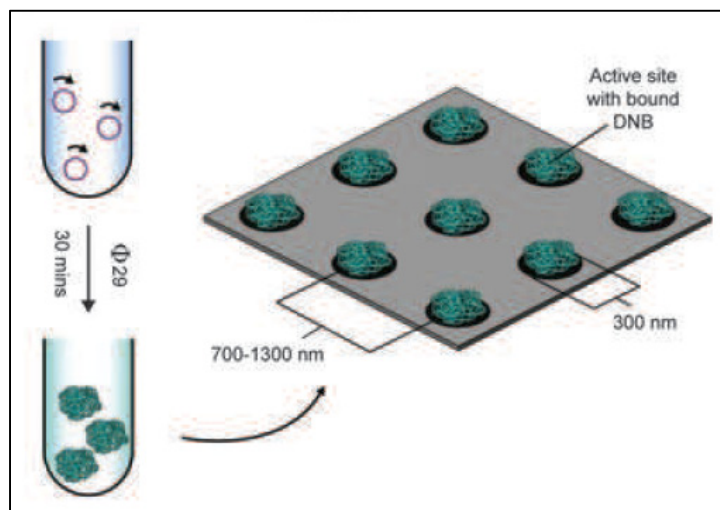


EX2048, Fig. 3 (cropped).

191. Drmanac 2010 further taught that because these Complete Genomics' DNA nanoballs were very large, they could only be sequenced using Complete Genomics' arrays with wells that were at least 300 nm in diameter. EX2047, 1, 3. Given that the radius for the Complete Genomics' arrays is 150 nm and the radius of the ZMW arrays is 35 nm, a POSA would have understood that the Complete Genomics' arrays have an area nearly 18 times larger¹². EX2047, 1, 3; EX2031, 1

¹² Given that the area of a circle is πr^2 , the area of the Complete Genomics' array is $22,500 \pi$ and the area of the ZMW array is $1,225 \pi$, which is approximately 18 times smaller.

EX2030, 4.



EX2047, Fig. 1C; EX2002, Fig. 3A.

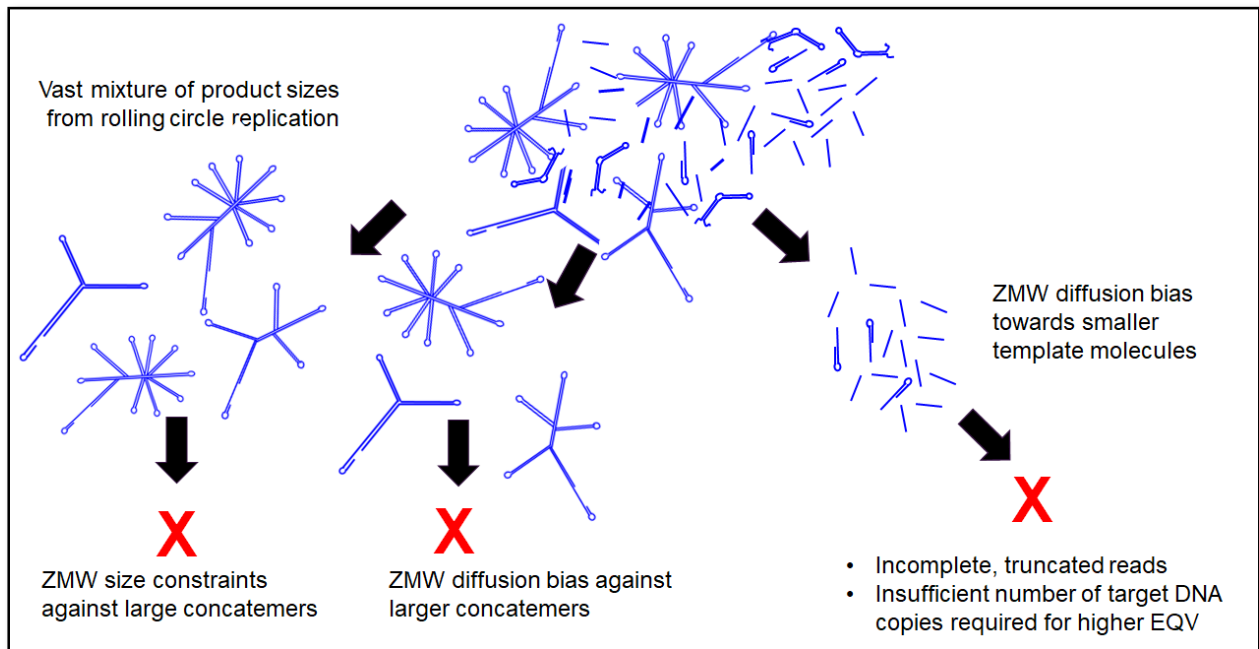
192. Moreover, Complete Genomics' system requires additional steps to sequence these large complex DNA nanoballs, which are absent from the SMRT sequencing workflow of Travers '075 and Travers 2010. For example, Stranneheim 2012 disclosed that sequencing Complete Genomics' large, complex DNA nanoballs required a special form of sequencing by ligation called "combinatorial probe-anchor ligation (cPAL) technology." EX2004, 1068; EX2002, 4332. Drmanac further taught that "cPAL is based on unchained hybridization and ligation technology." EX2047, 1. Neither Travers 2010 nor Travers '075 disclose the use of a different form of sequencing by ligation to accommodate the large size of the linear concatemers produced by Travers '075's rolling circle replication embodiment. Dr. Quackenbush has also not pointed to any

disclosures in either Travers '075 or Travers 2010 that teach additional processing steps to account for these large DNA products.

193. To sum up problems 1-4, a POSA would not have considered combining Travers '075's rolling circle replication embodiment with the SMRT sequencing of Travers '075 and Travers 2010. First, a POSA would have expected Travers '075's rolling circle replication embodiment to be unsequenceable in the ZMW arrays because each template would concurrently bind multiple primers and polymerase molecules. This would have created a problem with the single-molecule design of the SMRT™ sequencing workflow of Travers '075 and Travers 2010.

194. In addition, a POSA would have expected Travers '075's rolling circle replication embodiment to produce an array of reaction product sizes. As such, a POSA would have understood that the smaller concatemer products without template redundancy would outcompete the larger molecules to be sequenced in the ZMW wells. In addition, these small replication products would lack sufficient target DNA copies to achieve the required sequencing redundancy and EQV disclosed in Travers 2010. EX1021, Fig. 7. The large concatemer molecules produced from rolling circle replication would be outcompeted by the smaller products and would not easily fit into the narrow ZMW wells for SMRT™ sequencing.

195. As shown in the schematic summarizing these problems below, a POSA would understand that both small and large concatemer products from Travers '075's rolling circle replication embodiment are unusable.



196. As discussed above, Dr. Quackenbush does not address how the expected structures of the concatemers in Travers '075's rolling circle replication embodiment would overcome these issues, nor does he explain how a POSA would have resolved these critical problems. This comes as no surprise given that Dr. Quackenbush admitted that he did not consider DNA diffusion at all when preparing his declaration. EX2045, 15:24-25, 18:6-7, 18:16-17, 23:8-9, 24:1-2, 30:12-13, 31:12-18.

197. Moreover, a POSA would have understood that none of these

problems discussed above would have been present by using the circular SMRT sequencing applications of Travers 2010.

D. Dr. Quackenbush fails to explain *how* a POSA would have combined the rolling circle replication embodiment with the SMRT sequencing applications of Travers 2010.

198. Dr. Quackenbush fails to explain how a POSA would have practiced Travers '075's rolling circle replication embodiments. Specifically, Dr. Quackenbush does not explain how the long concatemer template molecules resulting from rolling circle replication are sequenced in the array of ZMW wells described in Travers 2010. Dr. Quackenbush argues that the library of long concatemers resulting from rolling circle replication can simply be added directly to the ZMW wells on the SMRT cell. EX1002, ¶57, 81. However, as I discussed above, there are at least four critical problems that Dr. Quackenbush does not address at all in his declaration.

199. *First*, Dr. Quackenbush does not explain *how* a POSA would have resolved the issue of sequencing multi-primed, multi-polymerase concatemer complex in the SMRT sequencing ZMW array. Again, Dr. Quackenbush does not explain how to resolve this issue.

200. *Second*, Dr. Quackenbush fails to explain how a POSA would have been able to sift through the broad smear of different-sized concatemer products from a rolling circle replication step to generate any useful sequencing readouts.

As discussed above, a POSA would have expected that rolling circle replication will produce a vast range of product sizes that accumulate over time. EX1021, Fig. 3; EX2036, Fig. 3B; EX2037, Fig. 6; EX2038, Fig. 3. Dr. Quackenbush does not address this problem in his declaration.

201. *Third*, Dr. Quackenbush does not address *how* a POSA would have added the long, linear rolling circle replication concatemer products to the ZMW array for the SMRT sequencing applications of Travers 2010. Travers '075 and Travers 2010 disclose only methods on how to conduct single-molecule sequencing using SMRTbell templates without an intervening amplification step. EX1021, 2; EX1018, ¶¶[0140]-[0141], [0146]. Indeed, neither reference provides any disclosures as to how a POSA would be able to perform SMRT sequencing using Dr. Quackenbush's proposed rolling circle replication concatemers as templates instead of the usual circular SMRTbell templates.

202. *Fourth*, Dr. Quackenbush does not explain *how* a POSA would have accounted for the well-known diffusion issues in the SMRT sequencing on ZMWs in order to sequence long, linear concatemers. As I discuss above in detail, a POSA would have known that there was a bias toward sequencing smaller DNA templates in the SMRT sequencing workflow. In addition, a POSA would have understood that the narrow diameter of the ZMWs creates a diffusion bias towards smaller DNA templates. However, it was also understood that the smaller

concatemer templates would possess fewer copies of the target DNA, which would not provide the required sequencing redundancy to achieve higher EQV and thus more reliable sequencing outputs. Moreover, as discussed above, these smaller, truncated templates would not have met the claim limitations that require confirming the presence of a sequence read from each of the first and second strands. Again, Dr. Quackenbush has admitted that he did not consider diffusion issues at all when preparing his declaration. EX2045, 15:24-25, 18:6-7, 18:16-17, 23:8-9, 24:1-2, 30:12-13, 31:12-18.

203. *Lastly*, Dr. Quackenbush does not explain *how* a POSA would have resolved how to fit large, structurally complex concatemers generated from rolling circle replication into the narrow ZMW wells. As discussed above, a POSA would have known that the long, linear concatemer products would form complex secondary and tertiary structures that would not have fit within the narrow ZMWs in the SMRT sequencing of Travers '075 and Travers 2010. Dr. Quackenbush does not address how to resolve this issue in his declaration.

204. Even though Travers '075 cites to U.S. Pat. No. 7,476,503 (“the ’503 patent”) when explaining that rolling circle replication products can be directly sequenced¹³, the ’503 patent also does not provide any more detail on how to

¹³ Travers '075 cites U.S. Pat. No. 7,476,503 when disclosing that “[t]hese

perform Travers '075's rolling circle replication embodiment. Indeed, just like Travers '075 and Travers 2010, the '503 patent only discloses that the SMRT sequencing methods do not utilize a template amplification step. In fact, the '503 patent discloses that "[t]his method also allows the use of target nucleic acid molecules taken *directly* from a biological sample, *minimizing the need for* cloning, subcloning, or *amplification* of the target nucleic acids *before sequencing* can take place." EX2008, 23:51-54 (emphasis added). Moreover, the '503 patent discloses that "[i]n another preferred embodiment, *sequencing is performed with polymerases capable of rolling circle replication,*" which a POSA would understand to mean that the *sequencing* is performed with rolling circle replication, not template amplification prior to sequencing. EX2008, 33:24-25.

205. Similarly, Travers '075 cites U.S. Provisional Application No. 61/072,160 ("the '160 application") when disclosing that "duplication processes may be employed to produce multiple copies of a prepared circular construct...." EX1018, ¶[0122]. The '160 application also does not provide any more detail on how to perform Travers '075's rolling circle replication embodiment and merely discloses methods of producing various template formats for sequencing. In fact,

replicated products may then be employed as template molecules in a template dependent sequencing process...." EX1018, ¶[0122] (citing EX1020).

most of the methods disclosed involve complicated processes of ligating exonuclease-sensitive tags to one strand of DNA and exonuclease-resistant tags to the other strand of DNA, followed by exonuclease digestion to produce single-stranded templates for sequencing. EX2049, ¶[0005], claims 1, 3, 8. Moreover, Dr. Quackenbush apparently did not rely on this reference at all because he did not cite it in his declaration. EX2045, 11:22-12:2.

1. Travers '075 does not enable its rolling circle replication embodiment.

206. In addition to the many problems associated with combining Travers '075's rolling circle replication embodiment with Travers 2010's SMRT sequencing applications, Travers '075 also does not enable the rolling circle replication embodiment it mentions. This is especially the case in view of the multi-primed, multi-polymerase concatemer multi-complex issue presented as problem 1 above. Based on the absence of any details in Travers '075, Travers 2010, or the general knowledge in the art on how to go about practicing this embodiment, Travers '075 does not allow practicing a rolling circle replication embodiment without undue experimentation.

207. I understand that in order to show that an embodiment is enabled, the specification must teach a POSA how to make and use the embodiment without undue experimentation. I understand that the question of undue experimentation

involves consideration of a number of factors, called the *Wands* factors, including:

(i) the quantity of experimentation necessary, (ii) the amount of direction or guidance presented, (iii) the presence or absence of working examples, (iv) the nature of the invention, (v) the state of the prior art, (vi) the relative skill of those in the art, (vii) the predictability or unpredictability of the art, and (viii) the breadth of the claims.

208. As discussed above, Travers '075's rolling circle replication embodiment is plagued by uncertainties and unpredictable outcomes. Indeed, neither Travers '075 nor Travers 2010 provide any direction or guidance on how to practice Travers '075's rolling circle replication embodiment or resolve any of the problems presented above. None of the other references cited by Dr. Quackenbush provide illumination either.

209. In addition, there are no working examples in either Travers '075 or Travers 2010 that provide any guidance as to how to make and use Travers '075's rolling circle replication embodiment on a ZMW array. Additionally, as demonstrated in my in-silico modeling experiments discussed above, concatemer templates would not have even fit into the ZMWs used for SMRT sequencing. As such, applying Travers '075's rolling circle replication embodiment would require excessive and undue quantities of experimentation.

210. Thus, a POSA would have understood that Travers '075 fails to

adequately instruct a POSA regarding how to perform its rolling circle replication embodiment and therefore does not enable its relied-upon embodiment.

XII. A POSA WOULD NOT HAVE HAD A REASONABLE EXPECTATION OF SUCCESS IN COMBINING TRAVERS '075'S ROLLING CIRCLE REPLICATION EMBODIMENT WITH THE SMRT SEQUENCING APPLICATIONS OF TRAVERS 2010.

A. A POSA would have known that there was no reasonable expectation of successfully implementing Travers '075's rolling circle replication embodiment.

211. As discussed above, Travers '075's rolling circle replication embodiment introduces many problems to the SMRT sequencing workflow without providing any additional benefits. In addition, these problems would have eliminated any reasonable expectation of success of implementing this embodiment with the sequencing applications of Travers 2010. *See* Section XI.C.2-4.

212. Moreover, a POSA would have understood that to even “obtain first and second strand reads sequence reads” as recited in claim 1, a POSA would have had to obtain a rolling circle replicon that represented at least one full revolution around the circular molecule. Indeed, as discussed above, the product of at least one full loop around the circular molecule contains both the sense and antisense strands of the template. EX1002, ¶¶77-82. However, a POSA would have also understood that there is no way to monitor or control the rolling circle replication process. As such, essentially all such replicons would proceed at least slightly

further than exactly one full circle around the circular molecule. EX1021, Fig. 3.

When the products contain even slightly more than exactly one copy of the circular template, a POSA would appreciate that the templates would then contain multiple primer/polymerase binding sites. *See* Section XI.C.2. As discussed above (Section XI.C.2), these templates would be essentially worthless because the ZMW array in SMRT sequencing would not have been able to sequence a multi-primed, multi-polymerase concatemer complex. *See* Section XI.C.2.

213. Thus, taking into account the technical problems identified above, a POSA would not have reasonably expected to successfully combine Travers '075's rolling circle replication embodiment in the SMRT sequencing applications of Travers 2010.

B. The '127 Patent's rolling circle replication embodiment does not support Dr. Quackenbush's arguments.

214. Dr. Quackenbush cites the '127 patent's specification and a claimed embodiment in claim 10 to support his opinion that Travers '075's rolling circle replication embodiment works within the SMRT sequencing workflow of Travers '075 and Travers 2010. EX1002, ¶61, 151-154. However, the '127 patent's claimed embodiment in claim 10 is not limited to the Travers '075's rolling circle replication embodiment.

215. Indeed, when discussing hairpin adapters and circular templates, the

'127 patent discloses that “[r]ecognition sites for restriction endonuclease digestion could be engineered into these adapters ... if more convenient for subsequent steps.” EX1001, 11:15-18. Thus, the templates of the '127 patent could be further processed, not sequenced directly after rolling circle replication, as suggested by Dr. Quackenbush. However, Travers '075 and Travers 2010 do not disclose any of these downstream modification steps disclosed in the '127 patent. The '127 patent also discloses that amplified products are sequenced “using any *suitable* method known in the art.” EX1001, 15:33-39 (emphasis added). A POSA reviewing this disclosure would understand which sequencing methods were suitable for certain template preparation methods and not suitable for others. As discussed above, a POSA would have understood that rolling circle replication was not suitable in the SMRT sequencing methods.

216. In addition, claim 10 of the '127 patent does not require that the templates be sequenced only using the SMRT sequencing on ZMW wells disclosed in Travers '075 and Travers 2010. Thus, the '127 Patent's rolling circle replication embodiment does not somehow resolve the numerous problems associated with implementing such a rolling circle replication embodiment in the SMRT sequencing workflow, as suggested by Dr. Quackenbush.

XIII. A POSA WOULD NOT HAVE BEEN MOTIVATED TO COMBINE TRAVERS '075 AND TRAVERS 2010 WITH MCCLOSKEY TO ARRIVE AT CLAIMS 13-14 AND 29-30.

217. Claim 13 recites the following: “The method of claim 1, wherein the adapters comprise a Y-shape or a U-shape, and wherein the barcodes *comprise a degenerate or semi-degenerate sequence* containing between 3 and 20 nucleotide bases.” EX1001, claim 13 (emphasis added). Claim 14 depends from claim 13, and recites certain structural arrangements of the degenerate or semi-degenerate barcodes within the adapters. EX1001, claim 14. Claims 29 and 30 recite barcode sequences 6 nucleotides in length and 3-8 nucleotides in length, respectively. EX1001, claims 29-30.

218. Dr. Quackenbush argues that Travers '075 and Travers 2010 in view of McCloskey teach U-shaped adapters comprising a “degenerate or semi-degenerate sequence containing 3 and 20 nucleotide bases.” EX1002, ¶¶191-204. I disagree. Neither Travers 2010 nor Travers '075 discloses using barcodes with a degenerate or semi-degenerate sequence containing between 3 and 20 nucleotides, as claimed in claim 13. Dr. Quackenbush instead relies on the disclosures of McCloskey (EX1023) for degenerate and semi-degenerate barcode sequences. EX1002, ¶¶195-199. A POSA would not have had any reason to combine McCloskey with Travers '075 and Travers 2010.

219. A POSA would have understood that McCloskey deals *exclusively*

with PCR amplification, which a POSA would understand to involve adding barcodes to track and identify separate *PCR products* that are amplification products of the same original template molecule. A POSA would have understood that PCR products are separate molecules. For example, McCloskey discloses that “[t]he *present invention* relates to the field of *polymerase chain reaction (PCR) amplification* of nucleic acid templates, and other forms of sequence analysis, particularly to the use of barcodes and batch-stamps for verifying the *authenticity of PCR products* and other sequence information.” EX1023, ¶[0002] (emphasis added). McCloskey further teaches that “the bar-coded target DNA molecule is amplified using the polymerase chain reaction (PCR).” EX1023, ¶[0014].

220. In its working examples, McCloskey also discloses that the purpose of its invention related to PCR. *See e.g.*, EX1023, ¶[0048] (“PCR Reaction”), ¶[0049] (“Screening of *Bar-coded PCR Products.*”), ¶[0054] (“*authenticating a PCR product* from the FMR1 locus by *using a bar-coded oligonucleotide....*”), ¶[0065] (“*authenticating a PCR product* from the FMR1 locus by *using two bar-coded oligonucleotides....*”), ¶[0073] (“PCR Reaction”), ¶[0074] (“Screening of *Bar-coded PCR Products*”); EX1023, ¶¶[0078], [0084], [0085], [0092]-[0098].

221. A POSA would have had no reason to use McCloskey’s random barcodes, which McCloskey designed and used exclusively for amplicon products that are separate molecules, in Travers ’075’s rolling circle replication

embodiment. This is because Travers '075's rolling circle replicons contain all the target DNA copies physically linked together as a single concatemer molecule. In other words, McCloskey's barcodes, designed and used exclusively for amplicon products that are separate molecules (PCR products), are used for an entirely different purpose that would not align with Travers '075's rolling circle replicons. McCloskey does not teach using its disclosed PCR methods on copies that are physically connected to one another or how to modify its methods to align with replicons from Travers '075's rolling circle replication embodiment. As such, a POSA would not have wanted to use McCloskey's random barcodes to link amplified copies to their original parent strand as exemplified in Travers '075's rolling circle embodiment.

222. Dr. Quackenbush argues that a POSA would have used McCloskey's barcodes in Travers '075's rolling circle replication embodiment because Travers '075 discloses using index barcodes and McCloskey discloses using random barcodes for sample identification. EX1002, ¶198. Indeed, Dr. Quackenbush stated the following in his declaration: "A person of ordinary skill in the art would have been motivated to use the barcodes of McCloskey with the hairpin adapters of the Travers Publications to facilitate identification of unique double-stranded molecules. *Travers '075 suggests that barcodes may be used to facilitate identification of sample sequences relative to other sequences in the mixture and*

McCloskey indicates that random barcodes may be used for this purpose.”

EX1002, ¶198 (emphasis added); EX1018, ¶[0100]; EX1023, ¶[0021]. However, Dr. Quackenbush fails to mention that McCloskey’s methods are designed for PCR amplification, and provides no other reason to combine McCloskey’s methods with those of the Travers ’075’s rolling circle replication embodiment.

223. In addition, Dr. Quackenbush fails to recognize that claims 29 and 30 depend from claim 22 and each require grouping the strand sequence reads into families based on the “barcode sequence” *and* “DNA fragment-specific information.” EX1001, claims 29-30. Instead, Dr. Quackenbush focuses only on a 6-mer barcode in McCloskey and argues that a POSA would select a 6-mer from McCloskey “*if that length of barcode was sufficient to assign a unique identity to each target nucleic acid molecule in the sample.*” EX1002, ¶203 (emphasis added). Dr. Quackenbush completely ignores the requirement in claims 29-30 that the grouping step requires using the barcode sequence *and DNA fragment-specific information*.

224. As such, Dr. Quackenbush has not sufficiently shown that claims 13-14 and 29-30 are obvious in view of Travers ’075, Travers 2010, and McCloskey.

XIV. DR. QUACKENBUSH FAILS TO ADDRESS SECONDARY CONSIDERATIONS OF NONOBVIOUSNESS.

225. I understand that when considering the obviousness of an invention,

one should also consider whether there is any objective evidence that supports the non-obviousness of the invention. I understand that objective evidence of non-obviousness includes factors such as failure of others; unexpectedly superior results; skepticism followed by praise in the industry; long-felt but unmet, need; copying; and commercial success. Dr. Quackenbush failed to address objective indicia of nonobviousness in his declaration.

A. Industry Praise

226. I understand that praise from those skilled in the field to which the patent relates can provide objective evidence of nonobviousness.

227. Dr. Kirsch (EX2007) praises the '127 patent's claimed duplex sequencing methods for its advances over the prior art: "Based on the data presented in the article by Schmitt et al. [], the Gordian knot in sequencing accuracy has apparently been cut by the *duplex sequencing* approach." EX2007, 14289 (emphasis added). Dr. Kirsch posited that duplex sequencing may "pave the way to explore the full power of NGS" and may ultimately "change the history of NGS." EX2007, 14289. Dr. Kirsch further praised duplex sequencing and stated that the "enthusiasm [over duplex sequencing] is warranted because duplex sequencing reportedly reduces the error rate of NGS up to 10 million-fold." EX2007, 14289. Moreover, GEN Edge in 2020 praised duplex sequencing as a "one in (ten) million" technology thought to be a "game changer" in applications,

such as “tumor evolution, liquid biopsies, prenatal diagnosis, [and] fetal genetic health.” EX2065, 1.

228. Dr. Kirsch and GEN Edge’s industry praise have a nexus with the full scope of at least claims 2-9, 16, 18, and 19 of the ’127 patent. I understand that evidence of industry praise for the invention may support nonobviousness if the evidence has a nexus to the claims. I understand that a nexus needs to be reasonably commensurate with the scope of the claims. There is no doubt that the claims of the ’127 patent cover aspects of duplex sequencing, which is the basis of the foregoing evidence of industry praise. EX1001, claims 2-9, 16, 18, 19.

229. Dr. Kirsch praises Patent Owner’s duplex consensus sequencing method for the claimed features in claims 2, 3, and 9. Specifically, Dr. Kirsch stated that the method first “identif[ies] families of DNA molecules that share the same tag sequence on a single strand” and further “identifies the complementary DNA strand by searching for the complementary tag sequences.” EX2007, 14289. Similarly, GEN Edge discloses, “the technique *independently tracks both strands* of individual DNA molecules and *compares* the results to identify and eliminate errors.” EX2065, 1.

230. Dr. Kirsch and GEN Edge’s praise has nexus with the full scope of claims 2, 3, and 9. Specifically, claim 2 requires separate steps of (a) comparing the confirmed first strand sequence read and confirmed second strand sequence

read; (b) identifying nucleotide bases that are not consistent between the two sequence reads; (c) identifying the bases that are consistent between the two sequence reads; and (d) then categorizing the sequence variation as either a processing error or a true variant. EX1001, claim 2. Claims 3 depends from claim 2 and recites associating the two sequence reads using an adapter sequence, sequence length, sequence alignment information and original strand information prior to comparing the first and second strand sequence. EX1001, claim 3. Similarly, claim 9 is directed to determining nucleotide bases that are not complementary between the confirmed first and second strand sequence reads and identifying non-complementary bases as either experimental error or sites of DNA damage. EX1001, claim 9. Each of these claims is directed to methods of comparing the first strand sequence reads and second strand sequence reads, and determining nucleotide bases positions at which the strands are not complementary.

231. The praise identified above also has a nexus with the full scope of claims 4-8. Dr. Kirsch states that the duplex sequencing method involves identifying “families of DNA molecules that share the same tag sequence on a single strand” and then identifying the “complementary DNA strand by searching for the complementary tag sequences.” EX2007, 14289. GEN Edge states that “[e]rrors are recognized and removed by comparing the sequences of PCR duplicates within each strand group and then between partner strands to find and

discount mismatches, increasing the confidence in the authenticity of any mutations that remain.” EX2065, 1.

232. Claim 4 depends from claim 1 and recites generating a consensus sequence for a plurality of adapter-DNA molecules, wherein each consensus sequence comprises nucleotide bases at which both the confirmed first and confirmed second strand sequence reads are in agreement. EX1001, claim 4. Claim 5 depends from claim 4 and further recites aligning the consensus sequence to the reference sequence and identifying a variant or a mutation occurring at a particular position or region as a true variant or mutation. EX1001, claim 5. Claim 6 depends from claim 5 and recites comparing the consensus sequence to the reference sequence and identifying a mutation type for each of the true mutations. EX1001, claim 6. Claim 7 depends from claim 6 and recites that the mutation type can be a number of different mutations. EX1001, claim 7. Claim 8 depends from claim 4 and further recites identifying a nucleotide sequence occurring at a particular position or region as a true nucleotide sequence. EX1001, claim 8. Each of these claims is directed to generating consensus sequences by comparing the first strand sequence read and the second strand sequence read to identify mutations. Thus, the above-mentioned praise have nexus with claims 4-8.

233. The above evidence of industry praise also have a nexus with the full scope of claims 16, 18, and 19. Claim 16 depends from claim 1 and recites

associating the confirmed first and second strand sequence reads using one or more of an adapter sequence and original strand information. EX1001, claim 16.

Similarly, claim 18 recites relating the first and second strand sequence reads based on a barcode sequence, a fragment-specific feature, or a combination thereof, while claim 19 is directed to differentiating two strand sequence reads at least in part by the asymmetry of the asymmetrical double-stranded adapter-DNA molecules.

EX1001, claims 18-19. Dr. Kirsch praises Patent Owner's duplex consensus sequencing method for these same claimed features, stating that the methods "exploit[] the redundant sequence information contained in the complementary DNA strand of double stranded genomic DNA molecule" by "add[ing] a unique random yet complementary double-stranded nucleotide sequence to both strands of duplex DNA before amplification." EX2007, 14289. GEN Edge further disclosed that, in duplex sequencing, "an *asymmetric portion* of the adapters ... *distinguishes the strands* so that each can be recognized as the original reference or anti-reference strand of the duplex." EX2065, 1 (emphasis added). Thus, a POSA would understand that Dr. Kirsch and GEN Edge's industry praise have nexus with claims 16, 18, and 19.

234. The '127 patent teaches that "by individually tagging and sequencing each of the two strands of a double-stranded (or duplex) target nucleic acid molecule and comparing the individual tagged amplicons derived from one half of

a double-stranded complex with those of the other half of the same molecule,” duplex consensus sequencing dramatically “lowers the error rate of sequencing.” EX1001, 17:3-10. As discussed above, claims 2-9, 16, 18, 19 are directed to methods of tagging, differentiating sequencing reads, comparing, and distinguishing actual mutations from sequencing errors and other technical artifacts. As such, each of these claims are central to the duplex sequencing technology that both Dr. Kirsch and GEN Edge praise in their respective articles.

235. In sum, a POSA would understand that the foregoing evidence of industry praise has a nexus with claims 2-9, 16, 18, and 19 of the '127 patent.

B. Long-Felt but Unmet Need

236. I understand that evidence of a long-felt, unmet need exists when, (1) the art recognized a problem that had existed for a long time without a solution and (2) the claimed invention solves that problem.

237. There existed a long-felt, but unmet need for the ability to accurately detect low-frequency mutations without invasive procedures. Indeed, before the invention of the '127 patent, clinicians performed invasive bone marrow biopsies to obtain a sufficient cell sample size for DNA analysis with NGS. EX2051, 2. Prior to March 20, 2012, next-generation sequencing applications for clinical diagnostics were limited and riddled with “inherently high sequencing error rate[s] of 1%, which results in hundreds of millions of sequencing mistakes.” EX2007,

14289; EX2051, 2. However, this long-felt need was solved by the inventors of the '127 patent who “developed duplex sequencing technology to improve sequencing accuracy, making it possible to find low-frequency variants at a level of detection that *has not been achievable before*.” EX2052, 4 (emphasis added).

238. In fact, Dr. Salk noted that he wanted to “focus on something that would bring an immediate benefit by addressing this particular unmet need, which is one that he sees on a daily basis” as an oncologist at a University of Washington-affiliated VA hospital. EX2052, 5. Dr. Salk also explained how Twinstrand’s duplex sequencing “could be used in almost any application where detection of rare variants is useful” and that Duplex Sequencing “could detect the impact of environmental factors on DNA mutagenesis and recognize patterns of mutations that result from smoking, aflatoxin, aristolochic acid, urethane, etc.” EX2052, 5. In addition, Twinstrand’s Duplex Sequencing was the “first NGS technology able to directly measure ultra-rare mutations caused by chemical carcinogens, without requiring biological selection and is applicable *in vitro* and *in vivo*.” EX2053, 1.

239. As discussed above, claims 2-9, 16, 18, and 19 of the '127 patent cover specific aspects of duplex sequencing, which is the basis of the foregoing evidence of a long-felt but unmet need. Thus, a POSA would have understood that there was a long-felt but unmet need for the ability to accurately detect low-

frequency mutations without invasive procedures and that the invention of the '127 patent solved this long-felt, but unmet need with its claimed duplex sequencing technology.

C. Unexpectedly superior results

240. I understand that unexpected results exist when the claimed invention exhibits superior properties or advantages that a POSA would have found surprising or unexpected. I also understand that when the surprising results are of a different kind, not just of a different degree, the court is more likely to find that unexpected results exists. I further understand that evidence of unexpectedly superior results support nonobviousness when there is a difference between the results obtained and those of the closest prior art.

241. When compared to the closest prior art, identified by Dr. Quackenbush as the SMRT sequencing applications of Travers 2010, the '127 patent's duplex sequencing methods demonstrated unexpectedly superior results. Travers 2010 discloses that SMRT sequencing provides its highest empirical quality value (EQV) at 6× sequencing depth which provides an error detection of about 1×10^{-4} (0.0001). EX1021, Fig. 7. As discussed above, this means that the probability of an incorrect base call was nearly 1 in 10,000. EX1021, Fig. 7; EX2055, 1.

242. In contrast, embodiments of the '127 patent's duplex sequencing

methods have an exceedingly low “theoretical background error rate of less than one artifactual error per 10^9 nucleotides sequenced and thus allows rare variants in heterogeneous populations to be detected with unprecedented sensitivity.”

EX2050, 1. Moreover, Schmitt 2012 taught that based on the background error frequency of 3.4×10^{-5} from its disclosed single strand DNA sequencing experiments, “the error frequency of DCS can be approximated as... 3.8×10^{-10} .”

EX2050, 5. These surprising and dramatically superior results from the ’127 patent are *over six orders of magnitude greater* in sensitivity than the accuracy of the methods of Travers 2010, which is far more than a difference in degree. Indeed, this astonishingly low error rate allows for measuring “mutation frequencies as low as 5×10^{-8} in human nuclear DNA” and a “direct measurement of the mutation frequency for the M13 bacteriophage as 2.5×10^{-6} mutations per base pair.”

EX2062, 3.

243. Since this evidence relates to duplex sequencing, it also has a nexus to claims 2-9, 16, 18, and 19 of the ’127 patent covering duplex sequencing. EX1001, claims 2-9, 16, 18, and 19. Indeed, the features of at least these claims allow the unprecedented accuracy demonstrated in these research papers. EX2050, 3; EX2062, 2.

244. However, Dr. Quackenbush fails to address any of this evidence of praise, long-felt need, or unexpected results in his declaration.

I hereby declare that all statements made herein are made of my own knowledge and are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Respectfully submitted,

Rahul Satija

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