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Substitute for form 1449/PTO <h2 style="text-align: center;">INFORMATION DISCLOSURE STATEMENT BY APPLICANT</h2> <p style="text-align: center;">(Use as many sheets as necessary)</p>				Complete if Known	
				Application Number	16017340
				Filing Date	06-25-2018
				First Named Inventor	AMORESE; Doug
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	23	of	61	Attorney Docket Number	25115-755.302

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No. ¹	Foreign Patent Document	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T ⁶
		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
	072	WO-2008115185-A3	12-24-2008	NIMBLEGEN SYSTEMS INC [US], et al.		☐
	073	WO-2009053039-A1	04-30-2009	ROCHE DIAGNOSTICS GMBH [DE], et al.		☐
	074	WO-2009112844-A1	09-17-2009	STICHTING HET NL KANKER I [NL], et al.		☐
	075	WO-2009120372-A2	10-01-2009	PACIFIC BIOSCIENCES CALIFORNIA [US], et al.		☐
	076	WO-2009120372-A3	01-07-2010	PACIFIC BIOSCIENCES CALIFORNIA [US], et al.		☐
	077	WO-2009120374-A2	10-01-2009	PACIFIC BIOSCIENCES CALIFORNIA [US], et al.		☐
	078	WO-2009120374-A3	12-17-2009	PACIFIC BIOSCIENCES CALIFORNIA [US], et al.		☐
	079	WO-2010003153-A2	01-07-2010	LIFE TECHNOLOGIES CORP [US], et al.		☐
	080	WO-2010030683-A1	03-18-2010	ROSETTA INPHARMATICS LLC [US], et al.		☐

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	081	WO-2010039991-A2	04-08-2010	TEXAS A & M UNIV SYS [US], et al.		☐
	082	WO-2010063711-A1	06-10-2010	ANGELETTI P IST RICHERCHE BIO [IT], et al.		☐
	083	WO-2010064893-A1	06-10-2010	KEYGENE NV [NL], et al.		☐
	084	WO-2010115154-A1	10-07-2010	FLUIDIGM CORP [US], et al.		☐
	085	WO-2010129937-A2	11-11-2010	LIFE TECHNOLOGIES CORP [US], et al.		☐
	086	WO-2011003630-A1	01-13-2011	MAX PLANCK GESELLSCHAFT [DE], et al.		☐
	087	WO-2011009941-A1	01-27-2011	OLINK GENOMICS AB [SE], et al.		☐
	088	WO-2011032053-A1	03-17-2011	NUGEN TECHNOLOGIES INC [US], et al.		☐
	089	WO-2011156529-A2	12-15-2011	NUGEN TECHNOLOGIES INC [US], et al.		☐
	090	WO-2012103154-A1	08-02-2012	NUGEN TECHNOLOGIES INC [US], et al.		☐

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	091	WO-2013059746-A1	04-25-2013	NUGEN TECHNOLOGIES INC [US], et al.		☐
	092	WO-2013177220-A1	11-28-2013	SCRIPPS RESEARCH INST [US], et al.		☐
	093	WO-2013191775-A2	12-27-2013	NUGEN TECHNOLOGIES INC [US], et al.		☐
	094	WO-9207951-A1	05-14-1992	KOW YOKE WAH [US]		☐
	095	WO-9318052-A1	09-16-1993	ISIS PHARMACEUTICALS INC [US]		☐
	096	WO-9416090-A1	07-21-1994	MOLECULAR TOOL INC [US], et al.		☐
	097	WO-9640998-A1	12-19-1996	PIONEER HI BRED INT [US]		☐
	098	WO-9712061-A1	04-03-1997	EPICENTRE TECHNOLOGIES CORP [US]		☐
	099	WO-9725416-A2	07-17-1997	NYFOTEK AS [NO], et al.		☐
	100	WO-9725416-A3	10-02-1997	NYFOTEK AS [NO], et al.		☐

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	101	WO-9806736-A1	02-19-1998	LIFE TECHNOLOGIES INC [US]		☐
	102	WO-9838296-A1	09-03-1998	GESHER ISRAEL ADVANCED BIOTECS [IL], et al.		☐
	103	WO-9844151-A1	10-08-1998	GLAXO GROUP LTD [GB], et al.		☐
	104	WO-9910540-A1	03-04-1999	LOPEZ OSVALDO J [US], et al.		☐
	105	WO-9911819-A1	03-11-1999	PERKIN ELMER CORP [US]		☐
	106	WO-9942618-A1	08-26-1999	DADE BEHRING INC [US]		☐

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	001	AB Applied Biosystems. The solid 3 system enabling the next generation of science. Presentation. 2009.	☐	
	002	ADAMCZYK, et al. Synthesis of a Chemiluminescent Acridinium Hydroxylamine (AHA) for the Direct Detection of Abasic Sites in DNA. Org. Lett. 1999; 1(5):779-781.	☐	
	003	ADAMCZYK, et al. O-(Fluoresceinylmethyl) hydroxylamine (OFMHA): A Fluorescent Regent for Detection of Damaged Nucleic Acids. Bioorg. & Med. Chem. Lett. 1998; 8:3599-3602.	☐	
	004	ADESSI, et al., Solid phase DNA amplification: characterisation of primer attachment and amplification mechanisms. Nucleic Acids Research. 2000 Oct. 15. 28:(20): e87.	☐	
	005	Agilent Technologies. Agilent Technologies adds human exon kit to next-generation-sequencing target enrichment portfolio. GenomicsNews.com. Posted 2009 Sept. 23, 2009. Available at http://www.genomicsnews.com/index.aspx?ID=103607&sm=Agilent%20technologies%20adds%20human%20exo . Accessed 10/6/2009.	☐	
	006	AHMED. Sequencing of Low-Diversity Libraries. 02/28/2012. http://cofactorgenomics.com/sequencing-low-diversity-libraries/ .	☐	
	007	ALBERT, et al. Direct selection of human genomic loci by microarray hybridization. Nat Methods. 2007 Nov;4(11):903-5. Epub 2007 Oct 14.	☐	
	008	ALVARADO, et al. Multiplexed direct genomic selection (MDiGS): a pooled BAC capture approach for highly accurate CNV and SNP/INDEL detection. Nucleic Acids Res. 2014 Jun;42(10):e82. doi: 10.1093/nar/gku218. Epub 2014 Mar 20.	☐	
	009	AMORESE, et al. Improved pathogen sequencing of host-pathogen RNA-sequence. AGBT 2016. Advances in Genome Biology Technology Conference. February 10, 2016. Orlando, FL. Nugen Technologies. Poster Presentation. 2 pages.	☐	

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	010	ANISIMOVA, et al. Isolation, characterization and molecular cloning of duplex-specific nuclease from the hepatopancreas of the kamchatka crab. BMC Biochemistry. 2008 May 21. 9:14 doi10.1186/1471-2091-9-14.	☐
	011	ANTSON, et al. PCR-generated padlock probes detect single nucleotide variation in genomic DNA. Nucleic Acids Res. 2000 Jun 15;28(12):E58.	☐
	012	ANWAR, et al. A stem-loop-mediated reverse transcription real-time PCR for the selective detection and quantification of the replicative strand of an RNA virus. Anal Biochem. 2006 May 1;352(1):120-8. Epub 2006 Feb 17.	☐
	013	ARCHER, et al. Selective and flexible depletion of problematic sequences from RNA-seq libraries at the cDNA stage. BMC Genomics. 2014 May 26;15:401. doi: 10.1186/1471-2164-15-401.	☐
	014	Arraystar, Inc. Arraystar Directional RNA-seq Prep Kit (dUTP Based). Cat#: A1208. 04/08/2013.	☐
	015	AUSUBEL, et al. eds. Current Protocols in Molecular Biology. Wiley. 1987.	☐
	016	BAIRD, et al. Rapid SNP discovery and genetic mapping using sequenced RAD markers. PLoS One. 2008;3(10):e3376.	☐
	017	BALLESTAR, et al. Methyl-CpG-binding proteins. Targeting specific gene repression. Eur J Biochem 2001; 268:1-6.	☐
	018	BANGS LABORATORIES, Inc. TechNote 205 retrieved at: http://www.bangslab.com/technotes/205.pdf . Visited on July 16, 2003. (8 pages).	☐
	019	BASHIARDES, et al. Direct genomic selection. Nat Methods. 2005 Jan;2(1):63-9.	☐

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	020	BEUCAGE, et al. The functionalization of oligonucleotides via phosphoramidite derivative. Tetrahedron. 1993;49(10):1925-63.	☐
	021	BEIER, et al. HT sequencing in biomedicine - new approaches in preparing samples. LABORWELT. Jan. 9, 2008.	☐
	022	BEN-ARTZI, et al. Double-stranded RNA-dependent RNase activity associated with human immunodeficiency virus type 1 reverse transcriptase. Proc Natl Acad Sci U S A. 1992 Feb 1;89(3):927-31.	☐
	023	BENTLEY, D. R. Whole-genome re-sequencing. Curr Opin Genet Dev. 2006 Dec;16(6):545-52. Epub 2006 Oct 18.	☐
	024	BHATTACHARJEE, et al. Complementing next generation sequencing technologies with Agilent's SureSelect DNA capture array. Agilent. 2009 July 13.	☐
	025	BIBIKOVA, et al. Targeted chromosomal cleavage and mutagenesis in drosophila using zinc-finger nucleases genetics. Genetics. 2002 July. 161: 1169-1175.	☐
	026	Bioo Scientific. Illumina RNA-Seq Library Prep. Available at http://www.biooscientific.com/ProductsServices/NextGenSequencing/Illumina-Compatible/RNA-Seq.aspx . Accessed 06/16/2014.	☐
	027	Bioo Scientific. NEXTflex RNA-Seq Kit. Available at http://www.biooscientific.com/ProductsServices/NextGenSequencing/Illumina-Compatible/RNA-Seq/NEXTflex%E2%84%A2RNA-SeqKit.aspx . Accessed 06/16/2014.	☐
	028	BLOW, N. Genomics: catch me if you can. Nature Methods. 2009 July. 6:7.539-544.	☐

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	029	BORMANN, et al. Whole methylome analysis by ultra-deep sequencing using two-base encoding. PLoS One. 2010 Feb 22;5(2):e9320.	☐
	030	BORODINA, et al. A strand-specific library preparation protocol for RNA sequencing. Methods Enzymol. 2011;500:79-98. doi: 10.1016/B978-0-12-385118-5.00005-0.	☐
	031	BOTURYN, et al. A simple and Sensitive Method for in Vitro Quantitation of Abasic Sites in DNA. Chem. Res. Toxicol. 1999; 12:476-482.	☐
	032	BOTURYN, et al. Synthesis of Fluorescent Probes for the Detection of Abasic Sites in DNA. Tetrahedron. 1997; 53(15):5485-5492.	☐
	033	BOWER, et al. Targeted rapid amplification of cDNA ends (T-RACE)--an improved RACE reaction through degradation of non-target sequences. Nucleic Acids Res. 2010 Nov;38(21):e194. doi: 10.1093/nar/gkq816. Epub 2010 Sep 15.	☐
	034	BRADFORD, et al. A comparison of massively parallel nucleotide sequencing with oligonucleotide microarrays for global transcription profiling. BMC Genomics. 2010 May 5;11:282. doi: 10.1186/1471-2164-11-282.	☐
	035	BRIGGS, et al. Targeted retrieval and analysis of five Neandertal mtDNA genomes. Science. 2009 Jul 17;325(5938):318-21. doi: 10.1126/science.1174462.	☐
	036	BRILL et al. Synthesis of oligodeoxynucleoside phosphorodithioates via thioamidites. J. Am. Chem. Soc. 111:2321-2322 (1989).	☐
	037	BROUDE. Stem-loop oligonucleotides: a robust tool for molecular biology and biotechnology. Trends Biotechnol. 2002 Jun;20(6):249-56.	☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Application Number	16017340
		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
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NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
	038	BROWN, T.A. Ed. Molecular Biology, LabFax. Bios Scientific Publishers. Academic Press. 1991; pp. 147-148.	☐
	039	BUCHMAN, et al. Selective RNA amplification: a novel method using dUMP-containing primers and uracil DNA glycosylase. PCR Methods Appl. 1993 Aug;3(1):28-31.	☐
	040	BURROWS, et al. Oxidative Nucleobase Modifications Leading to Strand Scission. Chem Rev. 1998 May 7;98(3):1109-1151.	☐
	041	CALLOW, et al. Selective DNA amplification from complex genomes using universal double-sided adapters. Nucleic Acids Res. 2004 Jan 28;32(2):e21.	☐
	042	CAREY, et al. Human Apurinic/Apyrimidinic Endonuclease in Processive. Biochem. 1999; 38:16553-16560.	☐
	043	CARLSSON et al. Screening for genetic mutations. Nature 380(6571):207 (1996).	☐
	044	CHAN, et al. The biophysics of DNA hybridization with immobilized oligonucleotide probes. Biophys J. 1995 Dec;69(6):2243-55.	☐
	045	CHEN, et al. BisQC: an operational pipeline for multiplexed bisulfite sequencing. BMC Genomics. 2014 Apr 16;15:290. doi: 10.1186/1471-2164-15-290.	☐
	046	CHEN, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005 Nov 27;33(20):e179.	☐
	047	CHENCHIK, et al. Full-length cDNA cloning and determination of mRNA 5' and 3' ends by amplification of adaptor-ligated cDNA. Biotechniques. 1996 Sep;21(3):526-34.	☐

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	048	Clontech Laboratories, Inc. In-Fusion SMARTer Directional cDNA Library Construction Kit User Manual. Cat. No. 634933. Copyright 2013.	☐
	049	CNV detection by ion semiconductor sequencing. Life Technologies. 2014.	☐
	050	COFACTOR genomics. Directional RNA Sequencing. Available at http://cofactorgenomics.com/directional-rna-sequencing . Accessed 06/04/2014.	☐
	051	Combined search and examination report dated 4/24/2013 for GB1305340.	☐
	052	CRAIG, et al. Identification of genetic variants using bar-coded multiplexed sequencing. Nat Methods. 2008 Oct;5(10):887-93. doi: 10.1038/nmeth.1251. Epub 2008 Sep 14.	☐
	053	CROUCHER, et al. A simple method for directional transcriptome sequencing using Illumina technology. Nucleic Acids Res. 2009 Dec;37(22):e148.	☐
	054	DAHL, et al. Multigene amplification and massively parallel sequencing for cancer mutation discovery. Proc Natl Acad Sci U S A. 2007 May 29;104(22):9387-92. Epub 2007 May 17.	☐
	055	DAHL, et al. Multiplex amplification enabled by selective circularization of large sets of genomic DNA fragments. Nucleic Acids Res. 2005 Apr 28;33(8):e71.	☐
	056	DEMPCY et al. Synthesis of a thymidyl pentamer of deoxyribonucleic guanidine and binding studies with DNA homopolynucleotides PNAS USA 92:6097-6101 (1995).	☐
	057	DERISI et al. Use of cDNA microarray to analyse gene expression patterns in human cancer. Nature Genetics. 1996;14:457-460.	☐

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	058	Diagnosing problems with phasing and pre-phasing on Illumina platforms. Loman Labs. 11/21/2013. http://nickloman.github.io/high-throughput%20sequencing/2013/11/21/diagnosing-problems-with-phasing-and-pre-phasing-on-illumina-platforms/ .	☐
	059	DRESSMAN, et al. "Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations." PNAS, July 2003, Vol. 100. No. 15, 8817-8822.	☐
	060	DRMANAC, et al. Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. Science. 2010 Jan 1;327(5961):78-81. doi: 10.1126/science.1181498. Epub 2009 Nov 5.	☐
	061	EGHOLM et al. Peptide nucleic acids (PNA) oligonucleotide analogues with an achiral peptide backbone. J Am Chem Soc 114:1895-1897 (1992).	☐
	062	EGHOLM et al. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature 365(6446):566-568 (1993).	☐
	063	ERLANGER, et al. Antibodies Specific For Ribonucleosides And Ribonucleotides And Their Reaction With DNA. Proc Natl Acad Sci USA. 1964; 52:68-74.	☐
	064	ESTELLER, Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet. 2007 Apr;8(4):286-98. Epub 2007 Mar 6.	☐
	065	European office action dated 04/01/2011 for Application No. 03771533.1.	☐
	066	European search report and opinion dated 05/22/2015 for EP Application No. 12842163.3.	☐
	067	European search report and opinion dated 07/23/2015 for EP Application No. 13740653.	☐

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	068	European search report and opinion dated 09/01/2016 for EP Application No. 14764629.3.	☐
	069	European search report and opinion dated 11/28/2013 for EP Application No. 11793123.8.	☐
	070	European search report and search opinion dated 04/03/2013 for Application No. 10808789.1.	☐
	071	European search report dated 10/18/2007 for Application No. 3771533.1.	☐
	072	European search report dated 2/12/10 for Application No. 7810169.8.	☐
	073	European search report dated 3/29/10 for Application No. 4815722.6.	☐
	074	FADROSH, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome. 2014 Feb 24;2(1):6. doi: 10.1186/2049-2618-2-6.	☐
	075	FAHY, et al., Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR. Genome Res. 1991. 1:25-33.	☐
	076	FAIRCLOTH, et al. Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. PLoS One. 2012;7(8):e42543. doi: 10.1371/journal.pone.0042543. Epub 2012 Aug 10.	☐
	077	FEINBERG, et al. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature. 1983 Jan 6;301(5895):89-92.	☐
	078	FODOR et al. Light-Directed, spatially addressable parallel chemical synthesis. Science. 1991;251:767-773.	☐

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	079	FRANCA, et al. Optimizing a qPCR gene expression quantification assay for S. epidermidis biofilms: a comparison between commercial kits and a customized protocol. PLoS One. 2012;7(5):e37480. doi: 10.1371/journal.pone.0037480. Epub 2012 May 21.	☐
	080	FRANK. BARCRAWL and BARTAB: software tools for the design and implementation of barcoded primers for highly multiplexed DNA sequencing. BMC Bioinformatics. 2009 Oct 29;10:362.	☐
	081	FREDRIKSSON, et al. Multiplex amplification of all coding sequences within 10 cancer genes by Gene-Collector. Nucleic Acids Res. 2007;35(7):e47. Epub 2007 Feb 22.	☐
	082	FREEMAN, et al. Fundamentals of DNA Hybridization Arrays for Gene Expression Analysis. BioTechniques. November 2000; 29:1042-1044, 1046, 1048-1055.	☐
	083	FRESHNEY, R.I. ed. (1987). Animal Cell Culture. IRL Press: Oxford, pp. vii-xii (Table of Contents Only).	☐
	084	FUJIWARA, et al. Direct probing: covalent attachment of probe DNA to double-stranded target DNA. Nucleic Acids Res. 1998 Dec 15;26(24):5728-33.	☐
	085	FULLWOOD, et al. Next Generation DNA sequencin of paired-end tags (PET) for transcriptome and genome analyses. Genome Research. 19(4):521-532 (April 2009).	☐
	086	GAIT, M.J., Ed. 1984 . Oligonucleotide Synthesis: A Practical Approach. IRL Press: Oxford, pp. vii-xii (Table of Contents).	☐
	087	GANOVA-RAEVA, et al. Primer Extension Enrichment Reaction (PEER): a new subtraction method for identification of genetic differences between biological specimens. Nucleic Acids Research. 2006; 34(11):e76.	☐

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	088	GERRISH, et al. Tailed pooled suppression subtractive hybridization (PSSH) adaptors do not alter efficiency. Antonie Van Leeuwenhoek. 2010 Nov;98(4):573-9. doi: 10.1007/s10482-010-9465-x. Epub 2010 Jun 8.	☐
	089	GERTZ, et al. Transposase mediated construction of RNA-seq libraries. Genome Res. 2012 Jan;22(1):134-41. doi: 10.1101/gr.127373.111. Epub 2011 Nov 29.	☐
	090	GHOSH, S.S. Synthesis of 5'-Oligonucleotide Hydrazide Derivatives and Their Use in Preparation of Enzyme-Nucleic Acid Hybridization Probes. Anal. Biochem. 1989; 178:43-51.	☐
	091	GNIRKE, et al. Solution Hybrid Selection with Ultra-long Oligonucleotides for Massively Parallel Targeted Sequencing. Nat. Biotechnol. (2009), 27(2):182-9.	☐
	092	GU, et al. Partitioning the c. elegans genome by nucleosome modification, occupancy, and position. Online 2009 August 25. http://www.springerlink.com/content/r0gw044155823242/fulltext.pdf . Accessed October 6, 2009.	☐
	093	GU, et al. Depletion of Abundant Sequences by Hybridization (DASH): using Cas9 to remove unwanted high-abundance species in sequencing libraries and molecular counting applications. Genome Biology. 2016; 17:41. DOI: 10.1186/s13059-016-0904-5.	☐
	094	GU, et al. Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. Nat Protoc. 2011 Apr;6(4):468-81. doi: 10.1038/nprot.2010.190. Epub 2011 Mar 18.	☐
	095	HARAGUCHI, et al. Synthesis and characterization of oligodeoxynucleotides containing formamidopyrimidine lesions and nonhydrolyzable analogues. J Am Chem Soc. 2002 Apr 3;124(13):3263-9.	☐
	096	HEIMGARTNER, et al. Polyacrylic Polyhydrazides as Reagents for Detection of Glycoproteins. Anal. Biochem. 1989; 181:182-189.	☐

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	097	HODGES, et al. Genome-wide in situ exon capture for selective resequencing. Nat Genet. 2007 Dec;39(12):1522-7. Epub 2007 Nov 4.	☐
	098	HODGES, et al. Hybrid selection of discrete genomic intervals on custom-designed microarrays for massively parallel sequencing. Nat. Protoc. 2009; 4(6): 960-974.	☐
	099	HOLLIS, et al. Structural studies of human alkyladenine glycosylase and E. coli 3-methyladenine glycosylase. Mutat Res. 2000; 460(3-4):201-10.	☐
	100	HORN, et al. Solid supported hydrolysis of apurinic sites in synthetic oligonucleotides for rapid and efficient purification on reverse-phase cartridges. Nucl. Acids Res. 1988; 16:11559-11571.	☐
	101	HOTTIGER, et al. Strand displacement activity of the human immunodeficiency virus type 1 reverse transcriptase heterodimer and its individual subunits. J Biol Chem. 1994 Jan 14;269(2):986-91.	☐
	102	HUBER, et al. Processing of the primer for plus strand DNA synthesis by human immunodeficiency virus 1 reverse transcriptase. J Biol Chem. 1990 Jun 25;265(18):10565-73.	☐
	103	HURD, et al. Advantages of next-generation sequencing versus the microarray in epigenetic research. Brief Funct Genomic Proteomic. 2009 May;8(3):174-83. doi: 10.1093/bfgp/elp013. Epub 2009 Jun 17.	☐
	104	IDE, et al. Synthesis and Damage Specificity of a Novel Probe for the Detection of Abasic Sites in DNA. Biochem. 1993; 32:8276-8283.	☐
	105	illumina Inc. Directional mRNA-Seq Sample Preparation - Application to prepare directional (strand specific) sample from mRNA. October 2010.	☐

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	106	International Preliminary Examination Report mailed on March 22, 2006 for PCT Patent Application No. PCT/US03/15825 filed May 19, 2003, 9pages.	☐
	107	International search report and written opinion dated 01/27/2012 for PCT Application No. US2011/039683.	☐
	108	International search report and written opinion dated 02/12/2013 for PCT/US2012/061218.	☐
	109	International search report and written opinion dated 02/24/2011 for PCT Application No. US10/55137.	☐
	110	International search report and written opinion dated 04/16/2013 for PCT Application No. US2013/023278.	☐
	111	International search report and written opinion dated 05/10/2012 for PCT Application No. US2012/22448.	☐
	112	International search report and written opinion dated 06/18/2015 for PCT/US2014/018112.	☐
	113	International search report and written opinion dated 07/15/2014 for PCT Application No. US2014/028356.	☐
	114	International search report and written opinion dated 07/29/2014 for PCT Application No. US2014/24581.	☐
	115	International search report and written opinion dated 10/18/2013 for PCT Application No. US2013/032606.	☐
	116	International search report and written opinion dated 12/3/2010 for PCT Application No. US10-45384.	☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Application Number	16017340
		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
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NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
	117	International search report dated 1/2/2008 for PCT Application No. US2007/15409.	☐
	118	International search report dated 6/14/2005 for PCT Application No. US 2003/015825.	☐
	119	International search report dated 7/9/2008 for PCT Application No. US2004/043710.	☐
	120	JENKINS et al. The Biosynthesis of Carbocyclic Nucleosides Chem Soc Re 24:169-176 (1995).	☐
	121	JONES, et al. The epigenomics of cancer. Cell. 2007 Feb 23;128(4):683-92.	☐
	122	KABOEY, et al. PCR hot start using primers with the structure of molecular beacons (hairpin-like structure). Nucleic Acids Res. 2000 Nov 1;28(21):E94.	☐
	123	KARATA, et al. Construction of a circular single-stranded DNA template containing a defined lesion. DNA Repair (Amst). 2009 Jul 4;8(7):852-6.	☐
	124	KAROW. New Capture Method Enables MPI Team to Sequence Five Neandertal Mitochondrial Genomes. GenomeWeb. July 21, 2009. https://www.genomeweb.com/sequencing/new-capture-method-enables-mpi-team-sequence-five-neandertal-mitochondrial-genom .	☐
	125	KAWARADA, et al. Antibodies Specific for Methylated DNA Elicited in Rabbits Recognize only a Single Strand Region of DNA Containing 7-Methylguanine. Tohoku. J Exp Med. 1986; 149:151-161.	☐
	126	KHRAPKO, et al. A method for DNA sequencing by hybridization with oligonucleotide matrix. DNA Sequence- J. DNA Sequencing and Mapping. 1991; 1:375-388.	☐
	127	KIEDROWSKI, et al. Parabolic growth of a self-replicating hexadeoxynucleotide bearing a 3'-5'-phosphoamidate linkage. Angew. Chem. Intl. Ed. English 1991;30:423-426.	☐

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	128	KIM, et al. Evidence for thiol-dependent production of oxygen radicals by 4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione (oltipraz) and 3H-1,2-dithiole-3-thione: possible relevance to the anticarcinogenic properties of 1,2-dithiole-3-thiones. Chem Res Toxicol. 1997 Mar;10(3):296-301.	☐
	129	KOSHKIN, et al. LNA (Locked Nucleic Acid): An RNA mimic forming exceedingly stable LNA:LNA duplexes. J. Am. Chem. Soc. 1998; 120:13252-3.	☐
	130	KOW, et al. Detection of Abasic Sites and Oxidative DNA Base Damage Using an ELISA-like Assay. Methods. 2000; 22:164-169.	☐
	131	KOZICH et al. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 79(17):5112-5120 (2013).	☐
	132	KRISHNAKUMAR, et al. A comprehensive assay for targeted multiplex amplification of human DNA sequences. Proc Natl Acad Sci U S A. 2008 Jul 8;105(27):9296-301. doi: 10.1073/pnas.0803240105. Epub 2008 Jul 2.	☐
	133	KRUEGER, et al. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics. 2011 Jun 1;27(11):1571-2. doi: 10.1093/bioinformatics/btr167. Epub 2011 Apr 14.	☐
	134	KRUEGER, et al. Large scale loss of data in low-diversity illumina sequencing libraries can be recovered by deferred cluster calling. PLoS One. 2011 Jan 28;6(1):e16607. doi: 10.1371/journal.pone.0016607.	☐
	135	KRUEGER. Loss of data in low-diversity libraries can be recovered by deferred cluster calling. Poster 01/29/2011. http://seqanswers.com/forums/showthread.php?t=9150 .	☐

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	136	KUBO, et al. A Novel Sensitive, and Specific Assay for Abasic Sites, the Most Commonly Produced DNA Lesion. Biochem. 1992; 31:3703-3708.	☐
	137	KUMAR, et al. A High-Throughput Method for Illumina RNA-Seq Library Preparation. Front Plant Sci. 2012 Aug 28;3:202. doi: 10.3389/fpls.2012.00202. eCollection 2012.	☐
	138	KURN. Method for generation of double stranded cDNA from RNA targets useful for global amplification, sequencing or other quantification of short RNA in a sample. March 21, 2010. 1-5.	☐
	139	LAIRD. Principles and challenges of genomewide DNA methylation analysis. Nat Rev Genet. 2010 Mar;11(3):191-203. doi: 10.1038/nrg2732.	☐
	140	LAO, et al. Real time PCR profiling of 330 human micro-RNAs. Biotechnol J. 2007 Jan;2(1):33-5.	☐
	141	LC Sciences. Targeted sequencing - sample enrichment service. 2009. Available at www.lcsciences.com/products/genomics/targeted_sequencing/targeted_sequencing.html . Accessed October 6, 2009.	☐
	142	LC Sciences. Technology - Massively parallel oligonucleotide and peptide synthesis on a microchip based on the uParaflo microfluidic technology. Available at www.lcsciences.com/support/technology/technology.html . Accessed October 6, 2009.	☐
	143	LC Sciences. Oligonucleotide mixture. OligoMix. 2009. Available at www.lcsciences.com/products/genomics/oligomix/oligomix_detail.html . Accessed October 6, 2009.	☐
	144	LEAMON, et al., a Massively parallel Pico TiterPlate based platform for discrete picoliter-scale polymerase chain reactions [abstract]. Electrophoresis. 2003 Nov. 24(21) 3769-77.	☐

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		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
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	145	LEFRANCOIS, et al. Efficient yeast ChIP-Seq using multiplex short-read DNA sequencing. BMC Genomics. 2009 Jan 21;10:37.	☐
	146	LENNON, et al. A scalable, fully automated process for construction of sequence-ready barcoded libraries for 454. Genome Biol. 2010;11(2):R15.	☐
	147	LEONARD. What is a reliable method for multiplexing more than 384 samples on a MiSeq run? Posted 08/19/2013. http://www.researchgate.net/post/What_is_a_reliable_method_for_multiplexing_more_than_384_samples_on_a_MiSeq_run2 .	☐
	148	LETSINGER et al. Cationic Oligonucleotides J Am Chem Soc 110:4470-4471 (1988).	☐
	149	LETSINGER et al. Effects of pendant groups at phosphorus on binding properties of d-ApA analogues. Nucl Acids Res 14(8):3487-3499 (1986).	☐
	150	LETSINGER et al. Phosphoramidate Analogs of Oligonucleotides J Org Chem 35(11):3800-3803 (1970).	☐
	151	LEVIN, et al. Comprehensive comparative analysis of strand-specific RNA sequencing methods. Nat Methods. 2010 Sep;7(9):709-15. doi: 10.1038/nmeth.1491. Epub 2010 Aug 15.	☐
	152	LHOMME, et al. Abasic DNA Structure reactivity and recognition. Biopolymers. 1999; 52(2): 65-83.	☐
	153	LI, et al. Targeted depletion of host reads in host-pathogen mixed RNA-seq libraries. Advances in Genome Biology Technology Conference. February 11, 2016. Orlando, FL. Nugen Technologies. Poster. 1 page.	☐

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				First Named Inventor	AMORESE; Doug
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Sheet	43	of	61	Attorney Docket Number	25115-755.302

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	154	LINDAHL, T. An N-Glycosidase from Escherichia coli That Releases Free Uracil from DNA Containing Deaminated Cytosine Residues. Proc Natl. Acad. Sci. USA 1974; 71(9):3649-3653.	☐
	155	LIZARDI, et al. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet. 1998 Jul;19(3):225-32.	☐
	156	LOCKHART, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nature Biotechnology, 14: 1675-1680 (1996).	☐
	157	MAG et al. Synthesis and selective cleavage of an oligodeoxynucleotide containing a bridged internucleotide 5'-phosphorothioate linkage. Nucleic Acids Res 19(7):1437-1441 (1991).	☐
	158	MAKRIGIOGOS, G. Fluorescent Labeling of Abasic Sites: A Novel Methodology to Detect Closely-Spaced Damage Sites in DNA. Int. J. Radiat. Biol. 1998; 74(1):99-109.	☐
	159	MARCHUK, et al. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. 1991 March 11; 19(5): 1154.	☐
	160	MARDIS, E. New strategies and emerging technologies for massively parallel sequencing: applications in medical research. Online 2009 April 17. Genome Med. 2009; 1(4): 40. Available at www.ncbi.nlm.nih.gov/pmc/articles/PMC2684661/?tool=pubmed . Accessed 10/22/2009.	☐
	161	MARDIS. Next-Generation DNA Sequencing Methods. The Annual Review of Genomics and Human Genetics. 2008; 9:387-402.	☐
	162	MARGULIES, et al. Genome sequencing in microfabricated high-density picolitre reactors [abstract]. Nature. 2005 Sep 15; 437 (7057): 376-80. Epub 2005 July 31.	☐

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	163	MASKOS et al. Oligonucleotide hybridisations on glass supports: a novel linker for oligonucleotide synthesis and hybridisation properties of oligonucleotides synthesised in situ. Nucleic Acids Research. 1992;20(7):1679-1684.	☐
	164	MAULIK, et al. Novel Non-isotopic Detection of MutY Enzyme-recognized Mismatches in DNA Via Ultrasensitive Detection of Aldehydes. Nucl. Acids. Res. 1999: 27(5):1316-1322.	☐
	165	MCCARTHY, et al. Inducible repair of O-alkylated DNA pyrimidines in Escherichia coli. EMBO J. 1984; 3(3):545-50.	☐
	166	MCCLURE, et al. Bovine exome sequence analysis and targeted SNP genotyping of recessive fertility defects BH1, HH2, and HH3 reveal a putative causative mutation in SMC2 for HH3. PLoS One. 2014 Mar 25;9(3):e92769. doi: 10.1371/journal.pone.0092769. eCollection 2014.	☐
	167	MCHUGH, et al. Novel Regents for Chemical Cleavage at Abasic Sites and UV Photoproducts in DNA. Nucl. Acids. Res. 23(10): 1664-1670.	☐
	168	MEIER et al. Peptide Nucleic Acids (PNAs)-Unusual Properties of Nonionic Oligonucleotide Analogues. Angew. Chem. Int. Ed. Engl.31(8):1008-1010 (1992).	☐
	169	MEISSNER, et al. Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. Nucleic Acids Res. 2005 Oct 13;33(18):5868-77. Print 2005.	☐
	170	METZKER, M. Sequencing technologies - the next generation. Nat Rev Genet. 2010 Jan;11(1):31-46. Epub 2009 Dec 8.	☐
	171	MEUZELAAR, et al. MegaPlex PCR: a strategy for multiplex amplification. Nat Methods. 2007 Oct;4(10):835-7. Epub 2007 Sep 16.	☐

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	172	MEYER, et al. Parallel tagged sequencing on the 454 platform. Nat Protoc. 2008;3(2):267-78. doi: 10.1038/nprot.2007.520.	☐
	173	MEYER, et al. Targeted high-throughput sequencing of tagged nucleic acid samples. Nucleic Acids Res. 2007;35(15):e97.	☐
	174	MITCHELL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008 Jul 29;105(30):10513-8.	☐
	175	MITRA, et al., In situ localized amplification and contact replication of many individual DNA molecules. Nucleic Acids Research. 1999. 27:(24); e34.	☐
	176	MITRA, et al. Oxidative DNA cleavage by the antitumor antibiotic leinamycin and simple 1,2-dithiolan-3-one 1-oxides: Evidence for thiol-dependent conversion of molecular oxygen to DNA-cleaving oxygen radicals mediated by polysulfides. Journal of the American Chemical Society. 1997; Vol.119(48):11691-11692.	☐
	177	MIZUGAKI, et al. Preparation of a monoclonal antibody specific for 5-methyl-2'-deoxycytidine and its application for the detection of DNA methylation levels in human peripheral blood cells. Biol Pharm Bull. 1996; 19(12):1537-1540.	☐
	178	MOLECULAR PROBE HANDBOOK Section 3.2 obtained from website at: http://www.probes.com/handbook/print/0302.html (Copyright© 1996-2003 by Molecular Probes, Inc.) Visited on August 13, 2003. (18 pages).	☐
	179	MULLIS, K.B et al., Eds. (1994). PCR: Polymerase Chain Reaction. Birkhauser: Boston, pp. xv-xvii (Table of Contents).	☐
	180	MYLLYKANGAS, et al. Efficient targeted resequencing of human germline and cancer genomes by oligonucleotide-selective sequencing. Nat Biotechnol. 2011 Oct 23;29(11):1024-7. doi: 10.1038/nbt.1996.	☐

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	181	NAKAMURA, et al. Highly Sensitive Apurinic/Apyrimidinic site Assay Can Detect Spontaneous and Chemically Induced Depurination Under Physiological Conditions. Cancer Res. 1998; 58:222-225.	☐
	182	NAYAK, et al. Functional architecture of T7 RNA polymerase transcription complexes. J. Mol Biol. 2007 August 10; 371(2): 490-500.	☐
	183	NEDDERMAN, et al. Cloning and expression of human G/T mismatch-specific thymine-DNA glycosylase. J Biol Chem. 1996; 271(22):12767-74.	☐
	184	New England BioLabs Inc. NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®. Available at https://www.neb.com/products/e7420-nebnext-ultra-directional-rna-library-prep-kit-for-illumina . Accessed 06/04/2014.	☐
	185	Nextera® Rapid Capture Enrichment Low-Plex Pooling Guidelines. Technical Note: DNA Analysis. 2014. http://www.illumina.com/content/dam/illumina-marketing/documents/products/technotes/technote-nextera-rapid-capture-low-plex-pooling-guidelines.pdf .	☐
	186	NEYLON, et al. Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for directed evolution. Nucleic Acids Res. 2004 Feb 27;32(4):1448-59. Print 2004.	☐
	187	NG, et al. Targeted capture and massively parallel sequencing of 12 human exomes. Nature. 2009 Sept. 10. 461, 272-276. http://www.nature.com/nature/journal/v461/n7261/full/nature08250.html . Accessed October 6, 2009.	☐
	188	NIKOLAEV, et al. Detection of genomic variation by selection of a 9Mb DNA region and high throughput sequencing. PLoS ONE. 2009 Aug. 17. 4(8): e6659.	☐

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Substitute for form 1449/PTO		Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Application Number	16017340
		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
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	189	Notice of allowance dated 07/28/2015 for US Application No. 13/643,056.	☐
	190	NUGEN, Inc. Ovation Biotin RNA Amplification and Labeling System User Guide. Catalog #2300-12. Published 2004.	☐
	191	NUGEN, Inc. Technical Report #1. The Ovation Biotin System Validation for Use with Affymetrix GeneChip Arrays. Published 2004.	☐
	192	OCHMAN, et al. Genetic applications of an inverse polymerase chain reaction. Genetics. 1988 Nov;120(3):621-3.	☐
	193	Office action dated 01/16/2013 for US Application No. 12/938,112.	☐
	194	Office action dated 02/08/2012 for EP Application No. 07810169.8.	☐
	195	Office action dated 02/17/2011 for US Application No. 12/305,633.	☐
	196	Office action dated 02/28/2013 for US Application No. 13/156,294.	☐
	197	Office action dated 03/01/2010 for US Application No. 10/441,663.	☐
	198	Office Action dated 03/03/2017 for US Application No. 15/154,414.	☐
	199	Office action dated 03/07/2007 for US Application No. 10/441,663.	☐
	200	Office action dated 03/09/2015 for CN Application No. 201380006942.4.	☐
	201	Office Action dated 03/15/2017 for US Application No. 14/390,012.	☐

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	202	Office action dated 04/03/2015 for CN Application No. 2012800608251.	☐
	203	Office action dated 04/16/2014 for US Application No. 13/239,226.	☐
	204	Office action dated 05/16/2011 for US Application No. 11/948,784.	☐
	205	Office action dated 05/25/2006 for US Application No. 10/441,663.	☐
	206	Office action dated 06/06/2012 for US Application No. 10/441,663.	☐
	207	Office action dated 06/19/2013 for US Application No. 12/855,611.	☐
	208	Office action dated 06/27/2013 for US Application No. 12/938,112.	☐
	209	Office action dated 06/30/2008 for US Application No. 11/026,280.	☐
	210	Office action dated 07/05/2007 for US Application No. 10/441,663.	☐
	211	Office action dated 07/08/2009 for US Application No. 10/441,663.	☐
	212	Office action dated 07/09/2015 for US Application No. 14/211,261.	☐
	213	Office action dated 07/13/2007 for US Application No. 11/026,280.	☐
	214	Office action dated 07/15/2008 for US Application No. 10/441,663.	☐
	215	Office action dated 07/15/2015 for US Application No. 13/938,059.	☐
	216	Office action dated 07/21/2016 for US Application No. 14/634,326.	☐

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		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
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	217	Office action dated 08/18/2010 for US Application No. 12/305,633.	☐
	218	Office action dated 09/05/2013 for US Application No. 13/156,294.	☐
	219	Office action dated 09/08/2016 for US Application No. 14/390,012.	☐
	220	Office action dated 09/09/2010 for US Application No. 10/441,663.	☐
	221	Office action dated 09/18/2006 for US Application No. 10/441,663.	☐
	222	Office action dated 09/24/2009 for US Application No. 10/441,663.	☐
	223	Office action dated 09/24/2014 for US Application No. 13/239,226.	☐
	224	Office action dated 10/09/2013 for US Application No. 12/938,112.	☐
	225	Office action dated 10/14/2010 for US Application No. 11/948,784.	☐
	226	Office Action dated 10-31-2016 for European Application 13806978.6.	☐
	227	Office action dated 11/04/2015 for US Application No. 14/030,761.	☐
	228	Office action dated 11/07/2012 for US Application No. 13/411,170.	☐
	229	Office action dated 11/13/2012 for US Application No. 12/855,611.	☐
	230	Office action dated 12/05/2008 for US Application No. 10/441,663.	☐
	231	Office action dated 12/17/2007 for US Application No. 10/441,663.	☐

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**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**

(Use as many sheets as necessary)

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Filing Date	06-25-2018
First Named Inventor	AMORESE; Doug
Art Unit	Not Yet Assigned
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	232	O'SHANNESY, et al. Immobilization of Glycoconjugates by Their Oligosaccharides: Use of Hydrazido-Derivatized Matrices. Anal. Biochem. 1990; 191:1-8.	☐
	233	OKOU, et al. Microarray-based genomic selection for high-throughput resequencing. Nat Methods. 2007 Nov;4(11):907-9. Epub 2007 Oct 14.	☐
	234	OLIVARIUS, et al. High-throughput verification of transcriptional starting sites by Deep-RACE. Biotechniques. 2009 Feb;46(2):130-2. doi: 10.2144/000113066.	☐
	235	OLSON, M. Enrichment of super-sized resequencing targets from the human genome. Nat Methods. 2007 Nov;4(11):891-2.	☐
	236	Openwetware. Directional-RNAseq Prep. Available at http://openwetware.org/wiki/Directional-RNAseq_Prep . Accessed 06/04/2014.	☐
	237	OYOLA, et al. Efficient Depletion of Host DNA Contamination in Malaria Clinical Sequencing. J. Clin. Microbiol. March 2013; 51(3):745-751.	☐
	238	PABINGER, et al. A survey of tools for variant analysis of next-generation genome sequencing data. Brief Bioinform. 2014 Mar;15(2):256-78. doi: 10.1093/bib/bbs086. Epub 2013 Jan 21.	☐
	239	PANG, et al. Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA. Molecular and Cellular Probes. 1992; 6:251-256.	☐
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	241	PARKHOMCHUK, et al. Transcriptome analysis by strand-specific sequencing of complementary DNA. Nucleic Acids Res. 2009 Oct;37(18):e123.	☐
	242	PAUWELS et al. Biological activity of new 2-5A analogues. Chemica Scripta 26:141-149 (1986).	☐
	243	PEASE, et al. A rapid, directional RNA-seq library preparation workflow for Illumina [reg] sequencing. Nature Methods. 2012; 9, no. 3.	☐
	244	PEASE et al. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. Proc. Natl. Acad. Sci. USA Biochemistry. 1994;91:5022-5026.	☐
	245	PEASE, et al. Rapid, directional RNA-seq library preparation kits for formalin-fixed paraffin-embedded RNA. Nature Methods. 2012; 9: Published online 27 September 2012.	☐
	246	PEI, et al. Site-specific cleavage of duplex DNA by semisynthetic nuclease via triple-helix formation. Pro. Natl. Acad. Sci. USA. 1990 Dec. 87: 9858-9862.	☐
	247	PENG, et al. Kamchatka crab duplex-specific nuclease-mediated transcriptome subtraction method for identifying long cDNAs of differentially expressed genes. Analytical Biochemistry. 2008 Jan. 15. 372:2, 148-155. (abstract).	☐
	248	PIERCE, et al. Linear-after-the-exponential polymerase chain reaction and allied technologies. Real-time detection strategies for rapid, reliable diagnosis from single cells. Methods Mol Med. 2007;132:65-85.	☐
	249	POLLACK, et al. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genet. 1999 Sep;23(1):41-6.	☐
	250	PORRECA, et al. Multiplex amplification of large sets of human exons. Nat Methods. 2007 Nov;4(11):931-6. Epub 2007 Oct 14.	☐

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	251	PRASHAR, et al. Analysis of differential gene expression by display of 3' end restriction fragments of cDNAs. Proc Natl Acad Sci U S A. 1996 Jan 23;93(2):659-63.	☐
	252	PROUDNIKOV, et al. Chemical methods of DNA and RNA fluorescent labeling. Nucleic Acids Res. 1996 Nov 15;24(22):4535-42.	☐
	253	RAMSAHOYE, et al. Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. Proc Natl Acad Sci U S A. 2000 May 9;97(10):5237-42.	☐
	254	RANASINGHE, et al. Fluorescence based strategies for genetic analysis. Chem Commun (Camb). 2005 Nov 28;(44):5487-502. Epub 2005 Sep 30.	☐
	255	RAWLS, Rebecca L. Optimistic about antisense. Promising clinical results and chemical strategies for further improvements delight antisense drug researchers. C & E News. 35-59 (Jun. 2, 1997).	☐
	256	RILEY, et al. A novel, rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. Nucleic Acids Res. 1990 May 25;18(10):2887-90.	☐
	257	ROBERTS, R. Restriction enzymes at NEB: over 30 years of innovation, the discovery, cloning and engineering of these essential reagents. NEB Expression. Winter. 2008. Vol 2.4. Available at www.neb.com/nebecomm/tech_reference/restriction_enzymes/feature_article_innovation.asp . Accessed 8/16/2010.	☐
	258	ROBERTSON. DNA methylation and human disease. Nat Rev Genet. 2005 Aug;6(8):597-610.	☐
	259	Roche Company. 454 life sciences, applications - sequence capture targeted region. http://www.454.com/applications/sequence-capture-targeted-region.asp . Accessed October 6, 2009.	☐

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	260	ROTHBERG, et al. An integrated semiconductor device enabling non-optical genome sequencing. Nature. 475 (2011): 348-352.	☐
	261	SAIKI, et al. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Nature. 1986 Nov 13-19;324(6093):163-6.	☐
	262	SAMBROOK et al. (eds.), Molecular Cloning—A Laboratory Manual, 1989, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. xi-xxxviii (Table of Contents Only).	☐
	263	SANDERS, et al. Targeting individual subunits of the FokI restriction endonuclease to specific DNA strands, Nucleic Acids Research. 2009 April. Nucleic Acids Res. 37:(7):2105-15.	☐
	264	SANGHVI, et al. ed. Chapters 2 and 3, ASC Symposium Series 580 - Carbohydrates Modifications in Antisense Research. American Chemical Society. Washington, DC. 1994.	☐
	265	SANO, et al. Detection of heavy methylation in human repetitive DNA subsets by a monoclonal antibody against 5-methylcytosine. Biochim Biophys Acta. 1988; 951(1):157-65.	☐
	266	SARTORI, et al. A novel uracil-DNA glycosylase with broad substrate specificity and an unusual active site. EMBO J. 2002; 21(12):3182-91.	☐
	267	SAWAI et al. Synthesis and Properties of Oligoadenylic Acids Containing 2'-5' Phosphoramidate Linkage. Chem. Lett. 13(5):805-808 (1984).	☐
	268	SCHENA, et al. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc Natl. Acad. Sci. USA Biochemistry. 1996; 93:10614-10619.	☐
	269	SCHENA et al. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. 1995;270:467-470.	☐

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Sheet	54	of	61

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Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
	270	SCHMID, et al. Chic and chec: genomic mapping of chromatin proteins. Molecular Cell. 2004. 16, no1, pp. 147-157. (abstract).	☐
	271	SEQanswers. MiSeq cluster generation problems. Posted 03/17/2012. http://seqanswers.com/forums/showthread.php?t=18499 .	☐
	272	SEQanswers. Sequencing a Low diversity library on the HiSeq. Posted 11/18/2011. http://seqanswers.com/forums/showthread.php?t=18499 .	☐
	273	SHALON et al. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Research. 1996;6(7):639-645.	☐
	274	SHENDURE, et al. Accurate multiplex polony sequencing of an evolved bacterial genome. Science. 2005 Sep 9;309(5741):1728-32. Epub 2005 Aug 4. Supplemental Materials. 41 pages.	☐
	275	SHIDA, et al. Cleavage of Single-and double-Stranded DNAs Containing an Abasic Residue by Escherichia coli Exonuclease III (AP Endonuclease VI) Nucl. Acids. Res. 1996; 24(22):4572-4576.	☐
	276	Singapore exam report dated 04/07/2015 for SG Application No. 11201404243W.	☐
	277	Singapore written opinion dated 03/17/2015 for SG Application No. 11201401628W.	☐
	278	SLUPPHAUG, et al. Low incorporation of dUMP by some thermostable DNA polymerases may limit their use in PCR amplifications. Anal. Biochem. 1993; 211:164-169.	☐
	279	SOHAIL, et al. Human activation-induced cytidine deaminase causes transcription-dependent, strand-biased C to U deaminations. Nucleic Acids Res. 2003; 31(12):2990-4.	☐

Examiner Signature	Date Considered
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Substitute for form 1449/PTO		Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Application Number	16017340
		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
Sheet	55	of	61

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Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
	280	SONI, et al. Progress toward ultrafast DNA sequencing using solid-state nanopores. Clin Chem. 2007 Nov;53(11):1996-2001. Epub 2007 Sep 21.	☐
	281	SPRINZEL et al. Enzymatic incorporation of ATP and CTP analogues into the 3' end of tRNA. Eur J Biochem 81(3):579-589 (1977).	☐
	282	SRIVASTAVA, et al. Mammalian Abasic Site Base Excision Repair. Identification of the Reaction Sequence and Rate-Determining Steps. J. Biol. Chem. 1998; 273(33):21203-21209.	☐
	283	STEMMER, et al. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. Gene. 1995 Oct 16;164(1):49-53.	☐
	284	STEPHPENS, et al. Automating sequence-based detection and genotyping of SNPs from diploid samples. Nat Genet. 2006 Mar;38(3):375-81. Epub 2006 Feb 19.	☐
	285	STEULLET, et al. Cleavage of Abasic Sites in DNA by Intercalator-amines. Bioorganic and Medicinal Chem. 1999; 7:2531-2540.	☐
	286	STEWART, et al. Complete MHC Haplotype Sequencing for Common Disease Gene Mapping. Genome Res. 2004 Jun;14(6):1176-87. Epub 2004 May 12.	☐
	287	Stratagene catalog, Gene Characterizatin Kits. 1988 page 39.	☐
	288	SUGIYAMA, et al. Chemistry of thermal degradation of abasic sites in DNA. Mechanistic investigation on thermal DNA stand cleavage of alkylated DNA. Chem. Res. Toxicol. 1994; 1:673-683.	☐
	289	SUMMERER, D. Enabling technologies of genomic-scale sequence enrichment for targeted high-throughput sequencing. Genomics. 2009 Dec;94(6):363-8. (abstract).	☐

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		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
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	290	SUMMERER, et al. Microarray-based muticycle-enrichment of genomic subsets for targeted next-generation sequencing. Accepted 2009 June 18. Available at www.ncbi.nlm.nih.gov/pubmed/19638418. Accessed 10/6/2009.	☐
	291	TEWHEY, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol. 2009 Nov;27(11):1025-31. doi: 10.1038/nbt.1583. Epub 2009 Nov 1.	☐
	292	TIMBLIN, et al. Application for PCR technology to subtractive cDNA cloning: identification of genes expressed specifically in murine plasmacytoma cells. Nucleic Acids Res. 1990 Mar 25;18(6):1587-93.	☐
	293	TONG, et al. Detection of restriction enzyme-digested target DNA by PCR amplification using a stem-loop primer: application to the detection of hypomethylated fetal DNA in maternal plasma. Clin Chem. 2007 Nov;53(11):1906-14. Epub 2007 Sep 27.	☐
	294	TUCKER, et al. Massively parallel sequencing: the next big thing in genetic medicine. Am J Hum Genet. 2009 Aug;85(2):142-54. doi: 10.1016/j.ajhg.2009.06.022.	☐
	295	TURNER, et al. Methods for genomic partitioning. Annu Rev Genomics Hum Genet. 2009;10:263-84. doi: 10.1146/annurev-genom-082908-150112. Review.	☐
	296	VAIRAPANDI, et al. Partial purification and characterization of human 5-methylcytosine-DNA glycosylase. Oncogene. 1996; 13(5):933-8.	☐
	297	VAIRAPANDI, et al. Human DNA-demethylating activity: a glycosylase associated with RNA and PCNA. J Cell Biochem. 2000; 79(2):249-60.	☐
	298	VARKONYI-GASIC, et al. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods. 2007 Oct 12;3:12.	☐

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		First Named Inventor	AMORESE; Doug
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		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
Sheet	57	of	61

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	299	VARLEY, et al. Nested Patch PCR enables highly multiplexed mutation discovery in candidate genes. Genome Res. 2008 Nov;18(11):1844-50. doi: 10.1101/gr.078204.108. Epub 2008 Oct 10.	☐
	300	VINCENT, et al. Helicase-dependent isothermal DNA amplification. EMBO Rep. 2004 Aug;5(8):795-800. Epub 2004 Jul 9.	☐
	301	VOS, et al. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 1995 Nov 11;23(21):4407-14.	☐
	302	WALKER et al. Strand displacement amplification--an isothermal, in vitro DNA amplification technique. Nucleic Acids Research 1992;20(7):1691-1696.	☐
	303	Westburg. Fast, Directional RNA-Seq Library Prep. Available at http://www.westburg.eu/lp/rna-seq-library-preparation . Accessed on 06/04/2014.	☐
	304	WESTIN, et al., Anchored multiplex amplification on a microelectronic chip array. Nature Biotechnology. 2000 Feb. 18(2):199-204.	☐
	305	Wikipedia. ABI solid sequencing. Http://en.wikipedia.org/wiki/ABI_Solid_Sequencing . Last modified 2009 Oct. 4. Accessed October 22, 2009.	☐
	306	Wikipedia. DNA sequencing. Available at http://en.wikipedia.org/wiki/Next-generation_sequencing . Last modified 2009 Oct. 8. Accessed 10/22/2009.	☐
	307	WILCHEK, et al. Labeling Glycoconjugates with Hydrazide Reagents. Methods Enzymol. 1987; 138:429-442.	☐
	308	WOLFFE, et al. DNA demethylation. Proc Natl Acad Sci USA. 1999; 96(11):5894-6.	☐

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		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
Sheet	58	of	61

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	309	WU, et al. Phasing Amplicon Sequencing For Robust Microbial Community Analysis. I-2630. 2014. http://www.asmonlineeducation.com/php/asm2014abstracts/data/papers/I-2630.htm .	☐
	310	XIAO, et al. Sequential amplification of flanking sequences by Y-shaped adaptor dependent extension using multiple templates. Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao (Journal Of Plant Physiology and Molecular Biology). 2007 Feb;33(1):85-90.	☐
	311	YOUNG, et al. A new strategy for genome assembly using short sequence reads and reduced representation libraries. Genome Res. 2010 Feb;20(2):249-56. doi: 10.1101/gr.097956.109.	☐
	312	ZALIPSKY, S. Functionalized Poly(ethylene glycol) for Preparation of Biologically Relevant Congugates. Bioconjugate Chem. 1995; 6:150-165.	☐
	313	ZANG, et al. DNA alkylation by leinamycin can be triggered by cyanide and phosphines. Bioorg Med Chem Lett. 2001 Jun 18;11(12):1511-5.	☐
	314	ZHANG, et al. Multiplex sequencing on the SOLID platform with 10, 16, or 96 barcodes. 2009 Life technologies. www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_065528.pdf .	☐
	315	ZHENG, et al. Titration-free 454 sequencing using Y adapters. Nat Protoc. 2011 Aug 18;6(9):1367-76. doi: 10.1038/nprot.2011.369.	☐
	316	ZHU, et al. Overexpression of 5-methylcytosine DNA glycosylase in human embryonic kidney cells EcR293 demethylates the promoter of a hormone-regulated reporter gene. Proc Natl Acad Sci USA. 2001; 98(9):5031-6.	☐
	317	ZHU, et al. 5-Methylcytosine DNA glycosylase activity is also present in the human MBD4 (G/T mismatch glycosylase) and in a related avian sequence. Nucleic Acids Res. 2000; 28(21):4157-65.	☐

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				Filing Date	06-25-2018
				First Named Inventor	AMORESE; Doug
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	59	of	61	Attorney Docket Number	25115-755.302

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	318	ZHULIDOV, et al. Simple cDNA normalization using kamchatka crab duplex=specific nuclease. Nucleic Acids Research.Online 2004 Feb. 18. 32:3 e37.	☐
	319	ZILLER, et al. Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. PLoS Genet. 2011 Dec;7(12):e1002389. doi: 10.1371/journal.pgen.1002389. Epub 2011 Dec 8.	☐

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		Filing Date	06-25-2018		
		First Named Inventor	AMORESE; Doug		
		Art Unit	Not Yet Assigned		
		Examiner Name	Not Yet Assigned		
Sheet	60	of	61	Attorney Docket Number	25115-755.302

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

- ☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

- ☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

- ☐ See attached certification statement.

- ☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

- ☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jeffrey Seidel/	Date (YYYY-MM-DD)	2018-12-04
Name/Print	Jeffrey Seidel	Registration Number	61,063

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875						Application or Docket Number 16/017,340			
APPLICATION AS FILED - PART I									
(Column 1)		(Column 2)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY			
FOR	NUMBER FILED	NUMBER EXTRA		RATE(\$)	FEE(\$)	RATE(\$)	FEE(\$)		
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A		N/A	300		
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A		N/A	660		
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A		N/A	760		
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	*				x 100 =	0.00		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	0.00		
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00		
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00		
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL		TOTAL	1720		
APPLICATION AS AMENDED - PART II									
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY	
AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT	MINUS	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE(\$)	ADDITIONAL FEE(\$)	RATE(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		x =	
	Application Size Fee (37 CFR 1.16(s))								
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))								
						TOTAL ADD'L FEE		TOTAL ADD'L FEE	
AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT	MINUS	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE(\$)	ADDITIONAL FEE(\$)	RATE(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		x =	
	Application Size Fee (37 CFR 1.16(s))								
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))								
						TOTAL ADD'L FEE		TOTAL ADD'L FEE	
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.									



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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
16/017,340	06/25/2018		1880	25115-755.302	20	1

12754

Wilson Sonsini Goodrich & Rosati/NUGEN
650 Page Mill Road
Palo Alto, CA 94304

CONFIRMATION NO. 2635
UPDATED FILING RECEIPT



0000000104209963

Date Mailed: 12/05/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Doug AMORESE, Los Altos, CA;
Chris ARMOUR, Kirkland, WA;
Nurith KURN, Palo Alto, CA;

Applicant(s)

NuGEN Technologies, Inc., San Carlos, CA;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 15/471,785 03/28/2017 PAT 10036012
which is a CON of 13/750,768 01/25/2013 PAT 9650628
which claims benefit of 61/591,241 01/26/2012

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 07/12/2018

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/017,340**

Projected Publication Date: 03/14/2019

Non-Publication Request: No

Early Publication Request: No

Title

COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT
AND HIGH EFFICIENCY LIBRARY GENERATION

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

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Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/017,340	06/25/2018	Doug AMORESE	25115-755.302

CONFIRMATION NO. 2635

PUBLICATION NOTICE



0000000106581679

12754
Wilson Sonsini Goodrich & Rosati/NUGEN
650 Page Mill Road
Palo Alto, CA 94304

Title: COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION

Publication No. US-2019-0078082-A1

Publication Date: 03/14/2019

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently <http://www.uspto.gov/patft/>.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

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Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

STATEMENT UNDER 37 CFR 3.73(c)Applicant/Patent Owner: NUGEN TECHNOLOGIES, INC.Application No./Patent No.: 16/017,340 Filed/Issue Date: June 25, 2018Titled: COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE
ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATIONNUGEN TECHNOLOGIES, INC., a Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☒ The assignee of the entire right, title, and interest.
2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):
- ☐ The extent (by percentage) of its ownership interest is _____ (%). Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.
- ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (e.g., bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

- A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 047636, Frame 0855, or for which a copy thereof is attached.
- B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

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The document was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.
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The document was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

STATEMENT UNDER 37 CFR 3.73(c)

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4. From: _____ To: _____

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Reel _____, Frame _____, or for which a copy thereof is attached.☐ Additional documents in the chain of title are listed on a supplemental sheet(s).☒ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Signature /Thomas C. Meyers/_____
Date May 17, 2019_____
Printed or Typed Name Thomas C. Meyers_____
Title or Registration Number 36,989

Electronic Acknowledgement Receipt

EFS ID:	36047938
Application Number:	16017340
International Application Number:	
Confirmation Number:	2635
Title of Invention:	COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION
First Named Inventor/Applicant Name:	Doug AMORESE
Customer Number:	12754
Filer:	Thomas C. Meyers/Michelle Aiello
Filer Authorized By:	Thomas C. Meyers
Attorney Docket Number:	25115-755.302
Receipt Date:	17-MAY-2019
Filing Date:	25-JUN-2018
Time Stamp:	16:37:47
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
------------------------	----

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	NuGEN-POA-091117.pdf	74250	no	1
			13b2c4ff177d4f8652d6c987cb3e90c4608850e7		

Warnings:

Information:					
2	Assignee showing of ownership per 37 CFR 3.73	NUGN-037-04US-Statement.pdf	26669	no	2
			e566b152f2363eeee8c29648b3ab9e084fff09f6		
Warnings:					
Information:					
Total Files Size (in bytes):			100919		
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					

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I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:



Practitioners associated with Customer Number:

21710

OR



Practitioner(s) named below (If more than ten patent practitioners are to be named, then a customer number must be used):

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Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignments documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:



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OR

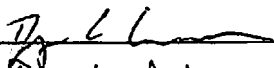
☐	Firm or Individual Name			
	Address			
	City		State	Zip
	Country			
	Telephone		Email	

Assignee Name and Address: NUGEN TECHNOLOGIES, INC.
201 INDUSTRIAL ROAD
SAN CARLOS, CA 94070

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of the practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature		Date	Sept 11, 2017
Name	Douglas A Amorese	Telephone	650 590-3615
Title	Chief Scientific Officer - NUGEN Technologies		

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/017,340	06/25/2018	Doug AMORESE	25115-755.302

21710
BROWN RUDNICK LLP
ONE FINANCIAL CENTER
BOSTON, MA 02111

CONFIRMATION NO. 2635
POA ACCEPTANCE LETTER



Date Mailed: 05/22/2019

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 05/17/2019.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/dtdinh/



Espacenet

Bibliographic data: WO2009112844 (A1) — 2009-09-17**METHOD OF PREPARING AN SHRNA LIBRARY**

Inventor(s): TE RIELE HENRICUS PETRUS JOSEPH [NL]; WIELDERS CAMIEL LAMBERT CHRISTIAAN [NL] ± (TE RIELE, HENRICUS, PETRUS, JOSEPH, ; WIELDERS, CAMIEL, LAMBERT, CHRISTIAAN)

Applicant(s): STICHTING HET NL KANKER I [NL]; TE RIELE HENRICUS PETRUS JOSEP [NL]; WIELDERS CAMIEL LAMBERT CHRIST [NL]; DEAN JOHN PAUL [GB] ± (STICHTING HET NEDERLANDS KANKER INSTITUUT, ; TE RIELE, HENRICUS, PETRUS, JOSEPH, ; WIELDERS, CAMIEL, LAMBERT, CHRISTIAAN, ; DEAN, JOHN, PAUL)

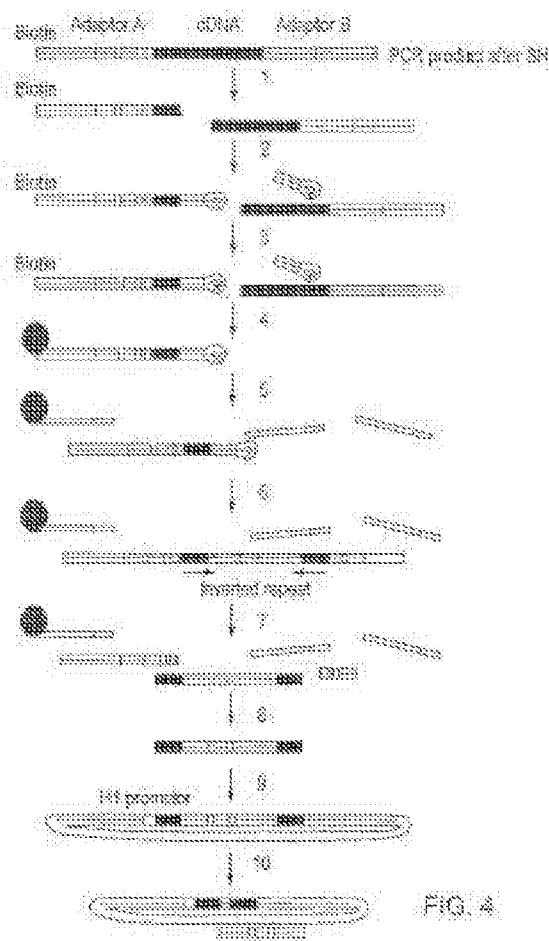
Classification: - **international:** **C12N15/10; C12N15/11**
- **cooperative:** **C12N15/1072; C12N15/111; C12N2310/14; C12N2330/31**

Application number: WO2009GB00684 20090313 Global Dossier

Priority number(s): GB20080004690 20080313

Abstract of WO2009112844 (A1)

There is provided a method of preparing an shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and second cell type, the method comprising the steps of: pre-identifying said one or more cDNA sequences using a first adaptor DNA sequence; and processing said one or more cDNA sequences using the first adaptor DNA sequence. There is further provided an shRNA library produced by such a method and uses thereof.





(43) International Publication Date
17 September 2009 (17.09.2009)

(10) International Publication Number
WO 2009/112844 A1

- (51) International Patent Classification:
C12N 15/10 (2006.01) *C12N 15/11* (2006.01)
- (21) International Application Number:
PCT/GB2009/000684
- (22) International Filing Date:
13 March 2009 (13.03.2009)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
0804690.6 13 March 2008 (13.03.2008) GB
- (71) Applicant (for all designated States except US):
**STICHTING HET NEDERLANDS KANKER INSTI-
TUUT** [NL/NL]; Plesmanlaan 121, NL-1066 CX Amster-
dam (NL).
- (71) Applicant (for LS only): **DEAN, John, Paul** [GB/GB];
Withers & Rogers, Goldings House, 2, Hays_Lane, Lon-
don SE1 2HW (GB).

- (72) Inventors; and
(75) Inventors/Applicants (for US only): **TE RIELE, Henri-
cus, Petrus, Joseph** [NL/NL]; Stichting Het Nederlands
Kanker Instituut, Plesmanlaan 121, NL-1066 CX Amster-
dam (NL). **WIELDERS, Camiel, Lambert, Christiaan**
[NL/NL]; Stichting Het Nederlands Kanker Instituut,
Plesmanlaan 121, NL-1066 CX Amsterdam (NL).
- (74) Agent: **DEAN, John, Paul**; Withers & Rogers, Goldings
House, 2, Hays_Lane, London SE1 2HW (GB).
- (81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ,
EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
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SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,

[Continued on next page]

(54) Title: METHOD OF PREPARING AN SHRNA LIBRARY

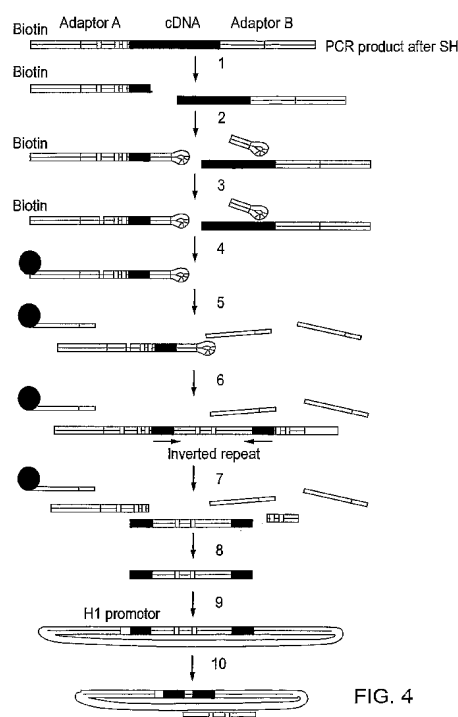


FIG. 4

(57) Abstract: There is provided a method of preparing an shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and second cell type, the method comprising the steps of: pre-identifying said one or more cDNA sequences using a first adaptor DNA sequence; and processing said one or more cDNA sequences using the first adaptor DNA sequence. There is further provided an shRNA library produced by such a method and uses thereof.

WO 2009/112844 A1



GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
- *with sequence listing part of description (Rule 5.2(a))*

METHOD OF PREPARING AN SHRNA LIBRARY

5 **Field Of The Invention**

The invention relates to low-complexity short hairpin RNA(shRNA) libraries and to a method of producing such a library. The libraries are useful in identifying genes which are responsible for phenotypic differences existing between two cell types among the genes which are differentially expressed genes in these cell types.

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Background

RNA interference (RNAi) has proved to be an important tool to knock-down gene function. Retro or lentiviral shRNA libraries targeting large parts of the human and murine genomes have been synthetically produced to study long-term effects of gene knock-down in genome-wide loss of function screens. Screening is much facilitated by a reduction of the library complexity, because fewer vectors require testing to cover the variety of library sequences, thus reducing the obligatory background. Therefore, smaller sub-libraries, for instance restricted to the deubiquitinating enzymes or the protein kinases, have been produced to reduce library complexity.

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Functional screens have become a very powerful tool to identify gene function. For gain-of-function screening, cDNA libraries were expressed in cells to identify genes whose over-expression affects the behaviour of cells. In this way, stimulatory genes (including oncogenes) have been discovered (e.g., Zhu *et al.* (2005) *Genetics* **170** 767-77; Staudt *et al.* (2005) *PLoS Genet.* Oct;1(4):e55 doi:10.1371/journal.pgen.0010055; Molnar *et al.*, (2006) *Genetics* **174** 1635-59; Mindorff *et al.* (2007) *Genetics* **176** 2247-63). These cDNA libraries, consisting of full-length cDNA copies of mRNA sequences, are enzymatically produced starting from RNA. Because RNA is a complex mixture, in which the number of mRNA copies of each gene varies enormously, this procedure yields very complex cDNA libraries. This means that many cDNA sequences need to be screened, in particular when the gene of interest is poorly expressed and therefore under-represented in the library. To overcome this problem, normalisation or subtractive hybridisation of cDNA preparations for gain-of-

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function screening have been used to reduce the complexity of cDNA libraries (Soares *et al.* (1994) Proc Natl Acad Sci USA **91** 9228-32; Diatchenko *et al.* (1996) Proc Natl Acad Sci USA **93** 6025-30; Perrett *et al.* (1998) Gene **208** 103-15; Fischer *et al.* (2003) Biotechniques **34** 250-4; Sternberg *et al.* (2007) Methods Mol Biol. **371** 289-305; Akopyants *et al.* (1995) Proc. Natl. Acad. Sci. USA **95** 13108-13113). Normalisation aims to equalize the full-length cDNA sequences, i.e., all genes present in the library are present in equal amounts. Subtractive hybridisation is used to select cDNA sequences that are differentially expressed between cell lines, in order to reduce the complexity of the resulting cDNA libraries even further.

More recently, the advent of RNA interference (RNAi) technology, which allows suppression of gene activity, has made it possible to also perform loss-of-function screens. RNAi is conferred by short (21 nucleotides) RNA sequences, which can either be directly introduced into cells as small interfering RNAs (siRNA), or be transcribed as short hairpins (shRNA) from expression vectors to obtain long-term RNAi. Several genome-wide shRNA libraries have been produced synthetically, i.e., by inserting custom-made oligonucleotide sequences into appropriate expression vectors. Screening of such libraries has identified a series of tumour suppressor genes (Bernards *et al.* (2006) Nat Methods **3** 701-6). Synthetic shRNA libraries are of course normalized, but are also very expensive to produce. Therefore, efforts have been undertaken to produce them enzymatically starting from RNA. Again, the complexity of the RNA presents a problem similar as for cDNA libraries. Therefore, Xu *et al.* (J. Biotechnol. (2007) **128** 477-485) described a protocol to enzymatically produce shRNA libraries from normalized RNA preparations. Although in these libraries all genes are more equally represented, they are still highly complex.

One way of generating low-complexity RNAi libraries only containing genes that are differentially expressed in the cell lines under study is to perform expression profiling and then select RNAi vectors targeting differentially expressed genes from a synthetic library. However, not every laboratory has easy access to a synthetic RNAi library or the technology to perform expression profiling.

Alternatively, subtractive hybridisation may be used to pre-select cDNA sequences that are differentially expressed in cell lines under study and use these sequences to produce RNAi libraries (Zhang *et al.* (2006) Stem Cells **24** 2661-2668). However, libraries produced in this way consisted of long double-stranded RNA molecules, which are considered to confer only a mild RNAi effect, inferior to that of shRNA vectors. Further, these long double stranded RNA molecules can induce an immune response in, for example, mammalian cells. This can cause non-specific gene silencing and apoptosis which is not desired.

10 Summary Of The Invention

According to a first aspect of the invention, there is provided a method of preparing an shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and second cell type, the method comprising the steps of: pre-identifying said one or more cDNA sequences using a first adaptor DNA sequence; and processing said one or more cDNA sequences using the first adaptor DNA sequence. Preferably, the first and second cell types are phenotypically different from one another. The first and second cell types may be of different cell strains or may be of the same cell strain.

20 The term “shRNA library” indicates a library of vectors each comprising a short inverted repeat cDNA sequence which, when transcribed, expresses an shRNA species which can be processed by a host cell to siRNA, able to interfere with the normal process of gene expression.

25 The term “enriched for one or more cDNA sequences” indicates that at least one cDNA sequence, corresponding to at least a portion of a gene which is differently expressed in a first and second cell type, is present in the shRNA library more frequently than in a normalised library in which all genes are equally represented, such as a library synthesised from oligonucleotides (e.g., Bernards *et al.* (2006) Nat. Methods **3** 701-6). Preferably, the shRNA library is enriched 2-500-fold, more preferably 50-200-fold, still more preferably 100-300-fold, most preferably about 200-fold for the one or more cDNA sequences. A 200-fold enrichment would indicate that at least one cDNA sequence, corresponding to at least a portion of a gene which

is differently expressed in a first and second cell type, is present in the shRNA library 200 times more frequently than in a normalised library in which all genes are equally represented. It is within the routine abilities of the skilled person to determine such levels of enrichment in a library according to the invention.

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Advantageously, the method according to the invention, including the initial step of pre-selection of putative target sequences, enables the production of very low-complexity shRNA libraries.

10 The term “pre-identifying said one or more cDNA sequences” indicates that genes which are differently expressed between the first and second cell types are initially selected, prior to the preparation of the shRNA library, with cDNA sequences corresponding to at least a portion of such genes being isolated.

15 In order to identify genetic differences between cell lines which are responsible for a selectable phenotypic difference, the library used for loss-of-function screening may be limited to the genes which are differentially expressed in these cells, because these especially are expected to contribute to the phenotypic change. For example, a low-complexity shRNA library to screen for tumour suppressor genes should primarily
20 consist of genes whose expression is reduced in tumour cells, to test if their knock-down results in proliferation.

Therefore, the method according to the invention produces shRNA libraries for loss-of-function screening, which are strongly enriched (for example, 50-200-fold) for
25 mRNA sequences down-regulated in the transcriptome associated with the selectable phenotype of interest compared to a control transcriptome (e.g., transformed vs. non-transformed cells). First, gene sequences are selected that are underrepresented in one transcriptome compared to another (for example, tumour suppressor genes), for example, using optimized PCR-based subtractive hybridisation. Subsequently, the
30 selected sequences, enriched for potential target sequences, are efficiently processed into a low-complexity shRNA library. The library can then be used in a loss-of-function screen for the selectable phenotype (e.g., cell proliferation), which can identify the genes responsible for the phenotypic transition.

shRNA libraries according to the invention can be produced for loss-of-function screening in a broad range of experimental settings and species to identify genetic differences between two cell line counterparts that differ by a selectable phenotype.

5 The shRNA libraries facilitate loss of function screening because they focus on putative target genes to reduce their complexity with respect to these genes. In addition, because the sequences which are pre-selected in the library are actually regulated during the phenotypic transition, any resulting "hits" identified by screening are likely to be relevant regulators of the phenotypic transition in the physiological
10 context of cells.

Advantageously, the method according to the first aspect of the invention combines the steps of pre-identifying cDNA sequences and processing them into an shRNA library into one integrated procedure. This is done by using the same adaptor DNA
15 sequence in both the first pre-identifying step and the second processing step. This reduces the complexity of the overall process and so the resulting method is simpler, easier to carry out and can be done in a relatively short period of time.

Preferably, in the method according to the first aspect of the invention, the step of pre-
20 identifying said one or more cDNA sequences comprises using subtractive hybridisation using the first adaptor DNA sequence and a second adaptor DNA sequence. The process of subtractive hybridisation which can be used to select gene sequences that are underrepresented in one transcriptome compared to another is well known to those skilled in the art, for example, see Diatchenko *et al.* (1996) Proc. Natl.
25 Acad Sci. USA **93** 6025-30. This method generally uses two adaptor DNA sequences which allow for PCR-based amplification of cDNA fragments that differ between a control and experimental transcriptome. The technique relies on the removal of dsDNA formed by hybridization between a control and test sample, thus reducing cDNAs of similar abundance, and retaining differentially expressed transcripts.

30 The two adaptor DNA sequences are generally ligated to cDNA fragments and allow the manipulation of the cDNA fragments. For example, if the adaptor DNA sequence comprises a marker, such as a stretch of adenine residues bound to Biotin, it allows

the isolation of cDNA fragments ligated to the adaptor DNA sequence. Each adaptor DNA sequence will comprise a primer binding site. This allows PCR amplification of the cDNA fragments. Preferably, Each adaptor DNA sequence has two different primer binding sites to allow nested PCR which reduces the contamination in products due to amplification of unwanted primer binding sites.

In a preferred embodiment of the invention, the pre-identifying step of the method comprises the steps of:

- a. carrying out a restriction digest reaction of cDNA from the first cell type using a first restriction enzyme;
- b. ligating the first adaptor DNA sequence, comprising a first primer recognition sequence and a second primer recognition sequence and a marker molecule, to DNA from step (a);
- c. ligating a second adaptor DNA sequence comprising a first primer recognition sequence and a third primer recognition sequence to DNA from step (a);
- d. carrying out a restriction digest reaction of cDNA from the second cell type using the first restriction enzyme;
- e. mixing the denatured products of step (b) with the denatured products of step (d), optionally in the presence of a size exclusion agent, in an annealing step;
- f. mixing the denatured products of step (c) with the denatured products of step (d), optionally in the presence of a size exclusion agent, in an annealing step;
- g. mixing the denatured products of steps (e) and (f) in the presence of a size exclusion agent in an annealing step, wherein the concentration of the size exclusion agent is higher than the concentration of the size exclusion agent in steps (e) and (f), if present;
- h. separating and isolating DNA carrying the first adaptor sequence from DNA carrying only the second adaptor sequence and/or no adaptor sequence, or separating and isolating DNA carrying the second adaptor sequence from DNA carrying only the first adaptor sequence and/or no adaptor sequence; and
- i. carrying out a nested PCR reaction using the separated DNA from (h) as template DNA and including the use of a first primer which recognises the first primer recognition sequence and, subsequently, a second primer which

recognises the second primer recognition sequence and a third primer which recognises the third primer recognition sequence.

The concentration of the size exclusion agent will depend on the particular size exclusion agent used. Preferably, the size exclusion agent is polyethylene glycol (PEG). Preferably, the concentration of size exclusion agent in steps (e) and (f), if present, is 1-10%. Preferably, the concentration of size exclusion agent in step (g) is 11-20%.

- 10 Having the concentration of size exclusion agent in step (g) higher than the concentration of size exclusion agent in steps (e) and (f) surprisingly causes a significant improvement in the efficiency of the pre-identifying step.

For the avoidance of doubt, not all of the steps need be carried out in the order given above; e.g., step (f) may be carried out before, or concurrently with, step (e). The term "comprises the steps of", as used throughout this specification, indicates that all steps are present but that their order need not be fixed. The skilled person would readily be able to determine a suitable alternative ordering to the steps set out above.

- 20 The first and second adaptor DNA sequences may comprise the nucleotide sequence:

GTATTACCGCACTCACTTGGAGCATCGTCCTGGCGTCTGGCTGAGGGTAGC
TGGAGAGGCGATCCGACCG (SEQ ID NO. 1), which may optionally be
hybridised to the sequence CGGTCGGATCGC (SEQ ID NO. 2); or

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AAAAAAAAAAAAAAAAAGTATTACCGCACTCACTTGGACTTCTGTCAC
CGTCACCGCATAGCTCATCTACGTCTTCC (SEQ ID NO. 3), which may
optionally be hybridised to the sequence GGAAGACGTAGATGAGC (SEQ ID NO.
4).

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The first and second adaptor sequences are preferably different to one another.

The first primer may have the nucleotide sequence:

GTATTACCGCACTCACTTGGA (SEQ ID NO. 5).

The second primer may have the nucleotide sequence:

CATCGTCCTGGCGTCTGGCT (SEQ ID NO. 6).

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The third primer may have the nucleotide sequence:

CTTCTGTCACCGTCACCGCATAG (SEQ ID NO. 7).

10 The effect of step (i) is that cDNA carrying both the first and second adaptor sequences is amplified by the PCR reaction at an exponential level, so the PCR product is significantly dominated by cDNA carrying both adaptors.

15 The first restriction enzyme is preferably an enzyme having a recognition site an average of once per 250 nucleotides in a typical genomic DNA sequence, most preferably *AluI*. An advantage of *AluI* is that the shRNA hairpins formed start with GCT (because this is a part of the *AluI* recognition sequence, which is AGCT). This is important for their functionality. Other suitable enzymes include: *MspI*, *HpaII*, *BstUI*, *HinPII*, *HhaI*, *HaeIII*, *PhoI*, *CviKI-1*, *TaqI*, *HpyCH4V*, *HpyCH2IV*.

20 Preferably, the marker molecule may be any molecule which enables identification and/or separation of cDNA carrying the first adaptor sequence, for example, biotin or streptavidin. The identification of DNA carrying the first adaptor sequence, in step (h), may be carried out by use of the marker molecule, e.g., where the marker molecule is biotin, DNA carrying the marker may be separated by binding to
25 streptavidin. Such methods are within the capabilities of the skilled person, who would be able to put them into effect without difficulty. Preferably, steps (e) and (f) of the method each continue for between 6 and 50 hours, more preferably between 40 and 50 hours, most preferably about 45 hours. In a further preference, step (g) of the method continues for about 24 hours. Preferably, the size exclusion agent (for
30 example, PEG) is present in steps (e) and (f) at 4-8%, more preferably at about 6% and in step (g) at 15-20%, preferably at about 17.5%. The separation of DNA in step (h) may be carried out by any of the means well known to the skilled person, for

example, by gel electrophoresis, band excision and purification, or using the marker molecule present on the first adaptor, for example by affinity separation on a column.

The method may further comprise the further step of inserting at least a portion of the PCR product from (i) into an shRNA expression vector.

In the method of the invention, once differentially expressed cDNAs have been pre-identified, they can be processed to produce inverted repeat sequences encoding for shRNA molecules directed to the at least one gene which is differently expressed in the first and second cell type. This is done, at least in part, using the first adaptor DNA sequence which is ligated to the one or more cDNA sequences.

The one or more cDNA sequences can be processed using: the first adaptor DNA sequence which comprises a recognition site for a first restriction enzyme; and a third hairpin adaptor DNA sequence to form hairpin structures, allowing the formation of inverted repeats.

Preferably, in the method of the invention, the processing step comprises the steps of:

- a. digesting the one or more cDNA sequences with a first restriction enzyme to produce a fragment of each of said one or more cDNA sequences attached to the first adaptor sequence;
- b. ligating the product of (a) with the third hairpin adaptor DNA sequence to form hairpin structures;
- c. replicating the hairpin structures of step (b) to form an inverted repeat of each of said one or more cDNA sequences;
- d. removing at least a portion the duplicated first adaptor DNA sequences from the product of step (c) using a second restriction enzyme; and
- e. processing the product of step (d), if necessary, to encode for a shRNA.

In step (a), the fragment of the cDNA sequence attached to the first adaptor sequence may be produced by the first restriction enzyme recognising a first recognition site in the first adaptor sequence and cleaving the cDNA attached to the first adaptor sequence at a distance from the first recognition site in the first adaptor sequence.

This would thus produce a cDNA fragment attached to the first adaptor sequence. The skilled person is well aware of restriction enzymes which cleave DNA at a distance from their recognition site, for example, *MmeI* (or another restriction enzyme which produces functional shRNA inserts, i.e., fragments which can be processed into shRNA molecules). The skilled person would have no problem in positioning the first recognition site in the first adaptor sequence in order to produce a fragment of the cDNA attached to the first adaptor sequence. The particular sequence of the first recognition site will depend on the particular restriction enzyme used and the skilled person would be able to determine this and position it accordingly. Preferably, the cDNA fragment attached to the first adaptor sequence after cleavage with the restriction enzyme is between about 10 and about 30 nucleotides in length. More preferably, the cDNA fragment is between about 15 and about 25 nucleotides in length and, most preferably, between about 18 and about 25 nucleotides in length.

In step (b), the third hairpin adaptor DNA sequence is ligated to the fragment of the cDNA sequence which is attached to the first adaptor sequence. This forms a hairpin structure. The third adaptor sequence can be any sequence which allows the formation of hairpin structures. In order to do this, the third adaptor sequence will contain two substantially complementary sequences which can bind together to form a stable hairpin structure. The two sequences do not have to be entirely complementary in order to produce a stable hairpin structure. They may be at least 90% complementary or, more preferably, at least 95% complementary. A skilled person could readily determine a suitable sequence to give a hairpin structure. Preferably, each of the two substantially complementary sequences is between about 5 and about 50 nucleotides long, more preferably, between about 10 and about 40 nucleotides long, even more preferably, between about 20 and about 30 nucleotides long and, most preferably, about 25 nucleotides long.

In step (c), the hairpin structures are replicated to form an inverted repeat of each of said one or more cDNA sequences. This can be done in any suitable way. For example, a primer can bind to the denatured hairpin structure at a primer binding site on the first adaptor sequence. Starting from the primer, the rest of the sequence can be replicated using a polymerase, for example, the Klenow fragment of DNA

polymerase I, to produce an inverted repeat of the cDNA sequence. The primer binding site can be the same as one of the primer sites used in subtractive hybridisation or it can be different. A person skilled in the art would readily be able to determine suitable primers and binding sites.

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Alternatively, the first adaptor sequence may be nicked by a third restriction enzyme which recognises a third recognition site on the first adaptor sequence. The term 'nick' means to cut only one of the strands of a double stranded DNA sequence. Nicking restriction enzymes are well known to those skilled in the art, for example, 10 Nb.BbvC-I (or any other enzyme which produces nicks in DNA). Once the first adaptor sequence is nicked, the product can be subjected to conditions which cause the release of the nicked portion of the first adaptor sequence. A primer can then bind in the region from which the nicked portion of the first adaptor sequence was released and the hairpin structure can be replicated starting from this primer, thereby forming 15 an inverted repeat of each the cDNA sequences.

In step (d), at least a portion of the duplicated first adaptor DNA sequences are removed from the product of step (c) using a second restriction enzyme. Preferably, the whole duplicated first adaptor sequences are removed. The second restriction 20 enzyme recognises a second recognition site in the first adaptor sequence and cleave the first adaptor sequence from the cDNA sequence. A person skilled in the art would be able to design and position the second recognition site so that the second restriction enzyme cleaves the first adaptor sequence from the cDNA sequence. The second restriction enzyme may be *BpmI*.

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This leaves an inverted repeat of the cDNA sequence, the inverted repeat being separated by the hairpin forming structure of the third adaptor sequence. Upon transcription of this inverted repeat, a shRNA molecule will form which can interfere with gene expression.

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If necessary, in step (e), the inverted repeat of the cDNA sequence can be processed to encode for a functional shRNA. For example, a portion of the third adaptor sequence which separates the inverted repeat may be removed if it is too long. This can be done

by having two restriction recognition sites in the third adaptor sequence so that a portion of the third adaptor sequence is cleaved and which can then be removed. The remaining ends of the third adaptor sequence can then be ligated together. The two restriction recognition sites may be for the same restriction enzyme or for different restriction enzymes. Preferably, they are for the same restriction enzyme. A suitable restriction enzyme is *BsgI*.

Preferably, the inverted cDNA repeat is inserted into an shRNA expression vector.

- 10 In one embodiment, step (e) can be performed after the inverted cDNA repeat has been inserted into an shRNA expression vector.

When subtractive hybridisation is used for the pre-identifying step, as discussed above, both the first and second adaptor DNA sequences will be attached to the cDNA sequences due to PCR amplification in subtractive hybridisation. Accordingly, these first and second adaptor DNA sequences need to be removed during processing of the cDNA sequences into sequences which correspond to shRNAs. The advantage of the present method is that the first adaptor DNA sequence is used both for subtractive hybridisation and for the processing of the cDNA. This means that the first adaptor sequence does not have to be removed and an alternative adaptor sequence used in the processing step. This ensures that the method is relatively simple.

Preferably, the processed cDNA sequence which encodes for a shRNA is inserted into an shRNA expression vector. Suitable expression vectors are well known to those skilled in the art.

In a slight variation to the method of the processing step described above, the processing step may comprise the steps of:

- a. carrying out a restriction digest reaction of the one or more pre-identified cDNA sequences using the first restriction enzyme;
- b. ligating the product of (a) with the third hairpin adaptor DNA sequence to form hairpin structures;

- c. nicking the first adaptor sequence in the hairpin structure of step (b) using the third restriction enzyme;
- d. isolating the product of the reaction in step (c) by binding to a marker molecule of the first adaptor sequence;
- 5 e. heating the product isolated in (d) with a primer;
- f. extension of the primer by a polymerase to generate the inverted DNA sequence complementary to the product of (f); and
- g. isolating at least a portion of the generated DNA product by use of the second restriction enzyme and inserting the resulting DNA into an shRNA expression vector.

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The third hairpin DNA adaptor sequence may have the nucleotide sequence:

TTCAAGAGAACGCGTTGCACCGGTGCTGCACCGGTGCAGCGCGTTCTCTT
GAANN (where N is A, G, C, or T) (SEQ ID NO. 8); or

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TTCAAGAGAN₁N₂GCGTTGCACCGGTGCTGCACCGGTGCAGCGCn₂n₁TCTCT
TGAA N₃N₄ (where N₁, N₂, N₃, N₄, n₁ and n₂ are independently selected from A, G, C, and T, and n₁ is complementary to N₁ and n₂ complementary to N₂) (SEQ ID NO. 12).

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The primer used in the processing step may have the nucleotide sequence CATCGTCCTGGCGTCTGGC (SEQ ID NO. 9).

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The shRNA expression vector may be selected from, by way of non-limiting example, pRetroSuper, a retroviral or lentiviral vector, or a vector encoding inducible RNAi or microRNA primary transcripts. A final digestion with a fourth restriction enzyme, such as *BsgI*, removes part of the hairpin adaptor and, after religation of the vector, a functional shRNA vector is formed. The skilled person would readily be able to determine alternative suitable restriction enzymes to those mentioned above. Other enzymes which may be suitable are: *MspI*, *HpaII*, *BstUI*, *HinPII*, *HhaI*, *HaeIII*, *PhoI*, *CviKI-1*, *TaqI*, *HpyCH4V*, or *HpyCH2IV*.

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While normalisation alone generates libraries encompassing all genes expressed in a particular tissue or cell line (Xu *et al.*, 2004), the inventors have found a method to produce libraries of very low complexity, strongly enriched for the small subset of genes which are differentially expressed in two cell types that differ by a selectable phenotype. Because the libraries of the invention focus on the few hundreds of genes that are differently regulated, screening for the actual gene that underlies the phenotypic transition becomes very easy. For the procedure, adaptors have been designed that allow subtractive hybridisation combining elements of the PCR selection method and pull down of desired sequences captured by magnetic beads. By using the same adaptors and specific restriction enzymes, selected cDNA sequences can subsequently be processed into inverted repeats that are finally introduced into shRNA-expression vectors (pSUPER). It has been found that the efficiency of subtractive hybridisation is much increased when a first round of hybridisation is performed in a low concentration (1-10% v/v) of polyethylene glycol (PEG) or another size exclusion agent, yielding a normalized number of single strands once the reaction is at equilibrium. A second hybridisation at a higher PEG concentration (11-30% v/v) shifts the equilibrium and drives further formation of hybrids. Addition of PEG enabled the optimisation of the length of the first hybridization, ultimately leading to libraries that more directly reflect the relative expression levels.

The method provides a technical solution to the problem of high cost and high complexity of current shRNA libraries, which compromise efficient screening. This is achieved by combining subtractive hybridisation and enzymatic production of shRNA libraries. To enable this, newly designed adaptors are used, which direct a more effective subtractive hybridisation procedure, followed by a new, efficient and easy procedure to process the selected sequences into shRNA libraries. The complexity of the resulting libraries is decreased by focusing on relevant sequences only, thus facilitating the subsequent screening. Such a method to produce enzymatically low complexity shRNA libraries that are enriched for differentially expressed genes has not been described or suggested in the prior art.

A second aspect of the invention relates to adaptor DNA sequences which can be used in the method described above. Accordingly, there is provided a first adaptor

nucleotide sequence for use in preparing a shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and second cell type, the adaptor sequence comprising: a first primer binding site for PCR amplification of a cDNA attached thereto, for example, following subtractive hybridisation;

a first restriction recognition site for a first restriction enzyme to cause the cleavage of the cDNA attached to the first adaptor sequence into a fragment; and

a second restriction recognition site for a second restriction enzyme to cause the cleavage of the cDNA fragment from at least a portion of the first adaptor sequence.

The first adaptor sequence can be used in subtractive hybridisation to pre-identify the cDNAs with differential expression between two cell types. It is then used to process the cDNAs into an shRNA library.

It is well within the capability of a person skilled in the art to design a first primer binding site to bind a first primer to allow a cDNA attached to the first adaptor sequence to be amplified using PCR. Preferably, the first adaptor sequence comprises a second primer binding site for binding a second primer to allow nested PCR.

The first restriction recognition site is for a restriction enzyme which cuts the nucleotide sequence away from the recognition site, i.e. in the cDNA attached to the first adaptor sequence. This produces a fragment of the cDNA which is still attached to the first adaptor sequence. It is well within the capability of a person skilled in the art to select a suitable restriction enzyme, e.g. *MmeI*, and position the recognition site in the first adaptor sequence to cause cleavage of the cDNA attached to the adaptor sequence into an appropriately sized fragment.

Similarly, with regard to the second restriction recognition site, it is well within the capability of a person skilled in the art to select a suitable second restriction enzyme to recognise the second restriction recognition site and position the second recognition site in the first adaptor sequence to cause the cleavage of the cDNA fragment from at least a portion of the first adaptor sequence. For example, a suitable restriction

enzyme is *BpmI*. Preferably, the entire first adaptor sequence is cleaved from the cDNA.

Preferably, the first adaptor sequence is between about 20 and about 200 nucleotides in length. More preferably, the first adaptor sequence is between about 20 and about 120 nucleotides in length, more preferably still, the first adaptor sequence is between about 40 and about 100 nucleotides in length, even more preferably, the first adaptor sequence is between about 50 and about 90 nucleotides in length, more preferably still, the first adaptor sequence is between about 60 and about 80 nucleotides in length and, most preferably, the first adaptor sequence is about 70 nucleotides in length.

Preferably, the first restriction recognition site for the first restriction enzyme causes the cleavage of the cDNA attached to the first adaptor sequence into a fragment of between about 10 and about 30 nucleotides in length. More preferably, the cDNA fragment is between about 15 and about 25 nucleotides in length and, most preferably, between about 18 and about 25 nucleotides in length.

Preferably, the first adaptor sequence comprises a third restriction recognition site for a third restriction enzyme to introduce a nick into the first adaptor sequence. A skilled person is well aware of suitable nicking enzymes, e.g. *Nb.BvCI*. The nick in the first adaptor sequence provides an entry site for a polymerase, e.g. the Klenow fragment of DNA polymerase I. This allows the cDNA to be replicated into an inverted repeat when used in conjunction with the third hairpin adaptor sequence as described above.

The first adaptor sequence preferably has a third primer binding site for binding a primer to allow replication of the cDNA. When the first adaptor sequence is used in conjunction with the third hairpin adaptor sequence, the primer can be used to replicate the cDNA into an inverted repeat. When the first adaptor sequence comprises a third restriction recognition site for a third restriction enzyme to introduce a nick into the first adaptor sequence, the third primer binding site can be within the region of nucleotides which are exposed when the nicked portion of the first adaptor

sequence is released from the first adaptor sequence under suitable conditions, e.g. denaturing conditions.

5 The primer binding sites in the first adaptor sequence, and also in other adaptor sequences of the invention, are preferably between about 15 and about 30 nucleotides long and, more preferably, between about 18 and about 25 nucleotides long.

10 In one embodiment, the first adaptor sequence comprises a marker molecule. The marker molecule may be any molecule which enables identification and/or separation of the first adaptor sequence. Suitable marker molecules are well known to those skilled in the art, for example, biotin or streptavidin.

In one embodiment, the first adaptor sequence has the following sequence:

15 GTATTACCGCACTCACTTGGAGCATCGTCCTGGCGTCTGGCTGAGGGTAGC
TGGAGAGGCGATCCGACCG (SEQ ID NO. 1); or

20 The present invention also provides a second adaptor sequence which can be used in conjunction with the first adaptor sequence in subtractive hybridisation to pre-identify cDNAs with different expression between two cell types. The second adaptor sequence comprises a first primer binding site to bind a first primer for PCR amplification of a cDNA attached thereto, for example, following subtractive hybridisation. The first primer binding site of the second adaptor sequence can bind the same primer as the first primer binding site on the first adaptor sequence. Preferably, the second adaptor sequence comprises a second primer binding site for
25 binding a second primer to allow nested PCR. Preferably, the second primer binding site of the second adaptor sequence binds a different primer than the second primer binding site of the first adaptor sequence.

30 In one embodiment, the second adaptor sequence comprises a marker molecule. The marker molecule may be any molecule which enables identification and/or separation of the second adaptor sequence. This can be used in subtractive hybridisation.

Preferably, the second adaptor sequence is between about 20 and about 200 nucleotides in length. More preferably, the second adaptor sequence is between about 20 and about 120 nucleotides in length, more preferably still, the second adaptor sequence is between about 40 and about 100 nucleotides in length, even more preferably, the second adaptor sequence is between about 50 and about 90 nucleotides in length, more preferably still, the second adaptor sequence is between about 60 and about 80 nucleotides in length and, most preferably, the second adaptor sequence is about 70 nucleotides in length.

- 10 In one embodiment the second adaptor sequence has the following sequence:
AAAAAAAAAAAAAAAAAAAAAGTATTACCGCACTCACTTGGACTTCTGTCAC
CGTCACCGCATAGCTCATCTACGTCTTCC (SEQ ID NO. 3)

The present invention also provides a third hairpin adaptor sequence. This can be any suitable sequence which allows the formation of a hairpin structure. The hairpin adaptor sequence can form hairpin structures and allows the production of inverted cDNA repeats using the method described above. In order to do this, the third adaptor sequence comprises two substantially complementary sequences which can bind together to form a stable hairpin structure. A skilled person could readily determine a suitable sequence to form a hairpin structure. As discussed earlier, the two sequences do not need to be entirely complementary. Preferably, each of the two substantially complementary sequences is between about 5 and about 50 nucleotides long, more preferably, between about 10 and about 40 nucleotides long, even more preferably, between about 20 and about 30 nucleotides long and, most preferably, about 25 nucleotides long.

Preferably, the third adaptor sequence is between about 20 and about 200 nucleotides in length. More preferably, the third adaptor sequence is between about 20 and about 120 nucleotides in length, more preferably still, the third adaptor sequence is between about 30 and about 90 nucleotides in length, even more preferably, the third adaptor sequence is between about 40 and about 80 nucleotides in length, more preferably still, the third adaptor sequence is between about 50 and about 70 nucleotides in

length and, most preferably, the third adaptor sequence is about 55 nucleotides in length.

Preferably, the third hairpin adaptor sequence comprises two restriction recognition sites for a restriction enzyme. This allows a portion of the third hairpin adaptor sequence to be removed once the cDNA inverted repeat has been produced. In the method of the invention, a suitable amount of the third hairpin adaptor can be removed to allow the formation of functional shRNA molecules as the remaining portion of the third hairpin adaptor sequence forms the hairpin section of the shRNA.

Preferably, a portion of the third hairpin adaptor sequence is removed to leave between about 4 and about 15 nucleotides between the cDNA repeats and more preferably, between about 7 and about 11 nucleotides. Accordingly, a skilled person would be able to select a suitable restriction enzyme or enzymes and position the recognition sites to allow the third hairpin adaptor sequence to be processed into a suitable length for producing functional shRNAs. Preferably, the two restriction recognition sites are for the same restriction enzyme.

In one embodiment the third hairpin adaptor sequence has the following sequence:

TTCAAGAGAACGCGTTGCACCGGTGCTGCACCGGTGCAGCGCGTTCTCTT

GAANN (where N A, G, C, or T) (SEQ ID NO. 8); or

TTCAAGAGAN₁N₂GCGTTGCACCGGTGCTGCACCGGTGCAGCGCn₂n₁TCTCT

TGAAN₃N₄ (where N₁, N₂, N₃, N₄, n₁ and n₂ are independently selected from A, G, C, and T and n₁ is complementary to N₁ and n₂ complementary to N₂) (SEQ ID NO.

12);

According to a third aspect of the invention, there is provided a kit for preparing an shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and second cell type, the kit comprising a first adaptor sequence as defined above. This kit can be used in the method described above.

Preferably, the kit further comprises a second adaptor sequence as defined above for use in subtractive hybridisation. Preferably, the kit further comprises a third hairpin adaptor sequence as defined above.

- 5 Preferably, the kit further comprises suitable primers to bind to the primer binding sites on the first adaptor sequence and the second adaptor sequence.

The kit may further comprise other components, for example, chemical reagents, materials for carrying out PCR or restriction enzymes.

10

According to a one embodiment, there is provided a kit for use in the method according to the first aspect of the invention. The kit comprises a polynucleotide sequence comprising the sequence:

GTATTACCGCACTCACTTGGAGCATCGTCCTGGCGTCTGGCTGAGGGTAGC

- 15 TGGAGAGGCGATCCGACCG (SEQ ID NO. 1);

and may comprise one or more polynucleotide sequences comprising one or more of sequences:

CGGTCGGATCGC (SEQ ID NO. 2); or

AAAAAAAAAAAAAAAAAAAAAGTATTACCGCACTCACTTGGACTTCTGTCAC

- 20 CGTCACCGCATAGCTCATCTACGTCTTCC (SEQ ID NO. 3); or

GGAAGACGTAGATGAGC (SEQ ID NO. 4); or

GTATTACCGCACTCACTTGGGA (SEQ ID NO. 5); or

CATCGTCCTGGCGTCTGGCT (SEQ ID NO. 6); or

CTTCTGTCACCGTCACCGCATAG (SEQ ID NO. 7); or

- 25 CATCGTCCTGGCGTCTGGC (SEQ ID NO. 9); or

TTCAAGAGAACGCGTTGCACCGGTGCTGCACCGGTGCAGCGCGTTCTCTT

GAANN (where N is A, G, C, or T) (SEQ ID NO.8); or

TTCAAGAGAN₁N₂GCGTTGCACCGGTGCTGCACCGGTGCAGCGCn₂n₁TCTCT

TGAAN₃N₄ (where N₁, N₂, N₃, N₄, n₁ and n₂ are independently selected from A, G,

- 30 C, and T and n₁ is complementary to N₁ and n₂ complementary to N₂) (SEQ ID NO. 12);

or sequences complementary to any of the above sequences.

The skilled person would readily be able to determine alternative sequences which would enable the method to be performed, subject to the requirements of, for example, the restriction enzymes, primers and marker molecules to be used.

- 5 According to a fourth aspect of the invention, there is provided an shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and a second cell type. That is, sequences from one or more genes which are expressed to the same or substantially the same level in the first and second cell types are normalised in the library, while
10 differentially expressed genes are enriched.

- The term “enriched for one or more cDNA sequences” indicates that at least one cDNA sequence, corresponding to at least a portion of a gene which is differently expressed in a first and second cell type, is present in the shRNA library more
15 frequently than in a normalised library in which all genes are equally represented, such as a library synthesised from oligonucleotides (e.g., Bernards *et al.* (2006) Nat. Methods **3** 701-6). Preferably, the shRNA library is enriched 2-500-fold, more preferably 50-200-fold, still more preferably 100-300-fold, most preferably about 200-fold for the one or more cDNA sequences. A 200-fold enrichment would indicate
20 that at least one cDNA sequence, corresponding to at least a portion of a gene which is differently expressed in a first and second cell type, is present in the shRNA library 200 times more frequently than in a normalised library in which all genes are equally represented.

- 25 The shRNA library is obtainable by the method of the invention. Preferably, the shRNA library is obtained by the method of the invention.

In one embodiment, a kit may comprise the shRNA library.

- 30 Advantageously, with respect to differently expressed genes, which represent potential targets of screening, such a library has a much lower complexity than prior art libraries, such that genes which differ in expression in two different cell types by only a small amount can be selected and, therefore, their effects studied.

According to a fifth aspect of the invention, there is provided a method of determining the effects of reduction in expression of a gene of interest, comprising the use of an shRNA library according to the fourth aspect of the invention.

5

According to a another aspect of the invention, there is provided an isolated polynucleotide sequence comprising any one of sequences:

GTATTACCGCACTCACTTGGAGCATCGTCCTGGCGTCTGGCTGAGGGTAGC
TGGAGAGGCGATCCGACCG (SEQ ID NO. 1); or

10 CGGTCGGATCGC (SEQ ID NO. 2); or

AAAAAAAAAAAAAAAAAAAAAGTATTACCGCACTCACTTGGACTTCTGTCAC
CGTCACCGCATAGCTCATCTACGTCTTCC (SEQ ID NO. 3); or

GGAAGACGTAGATGAGC (SEQ ID NO. 4); or

GTATTACCGCACTCACTTGGGA (SEQ ID NO. 5); or

15 CATCGTCCTGGCGTCTGGCT (SEQ ID NO. 6); or

CTTCTGTCACCGTCACCGCATAG (SEQ ID NO. 7); or

CATCGTCCTGGCGTCTGGC (SEQ ID NO. 9); or

TTCAAGAGAACGCGTTGCACCGGTGCTGCACCGGTGCAGCGCGTTCTCTT
GAANN (where N A, G, C, or T) (SEQ ID NO. 8); or

20 TTCAAGAGAN₁N₂GCGTTGCACCGGTGCTGCACCGGTGCAGCGC_{n₂n₁}TCTCT
TGAAN₃N₄ (where N₁, N₂, N₃, N₄, n₁ and n₂ are independently selected from A, G,
C, and T and n₁ is complementary to N₁ and n₂ complementary to N₂) (SEQ ID NO.
12);

25 or sequences complementary thereto.

The skilled person would readily be able to determine alternative sequences which would enable the method to be performed, subject to the requirements of, for example, the restriction enzymes, primers and marker molecules to be used.

30

According to a the pre-identification step of the invention, there is provided a method of identifying at least one gene which is differently expressed in a first and a second cell type, the method comprising the steps of:

- a. carrying out a restriction digest reaction of cDNA from the first cell type using a first restriction enzyme;
- b. ligating a first adaptor DNA sequence, comprising a first primer recognition sequence and a second primer recognition sequence and a marker molecule, to
5 DNA from step (a);
- c. ligating a second adaptor DNA sequence comprising a first primer recognition sequence and a third primer recognition sequence to DNA from step (a);
- d. carrying out a restriction digest reaction of cDNA from the second cell type using the first restriction enzyme;
- 10 e. mixing the denatured products of step (b) with denatured products of step (d), optionally in the presence of a size exclusion agent, in an annealing step;
- f. mixing the denatured products of step (c) with denatured products of step (d), optionally in the presence of a size exclusion agent, in an annealing
15 step;
- g. mixing the denatured products of steps (e) and (f) in the presence of a size exclusion agent in an annealing step, wherein the concentration of the size exclusion agent is higher than the concentration of size exclusion agent in steps (e) and (f);
- 20 h. separating and isolating DNA carrying the first adaptor sequence from DNA carrying only the second adaptor sequence and/or no adaptor sequence, or separating and isolating DNA carrying the second adaptor sequence from DNA carrying only the first adaptor sequence and/or no adaptor sequence;
- i. carrying out a nested PCR reaction using the separated DNA from (h) as
25 template DNA and including the use of a first primer which recognises the first primer recognition sequence and, subsequently, a second primer which recognises the second primer recognition sequence and a third primer which recognises the third primer recognition sequence; and
- j. separating and isolating the product(s) of the reaction in step (i), said product
30 being at least a fragment of a gene which is expressed at a lower level in the second cell type than in the first cell type.

Preferably, the concentration of size exclusion agent in steps (e) and (f), if present, is 1-10%. Preferably, the concentration of size exclusion agent in step (g) is 11-30%.

The first and second adaptor DNA sequences may have the nucleotide sequence:

5 GTATTACCGCACTCACTTGGAGCATCGTCCTGGCGTCTGGCTGAGGGTAGC
TGGAGAGGCGATCCGACCG (SEQ ID NO. 1), which may optionally be
hybridised to the sequence CGGTCGGATCGC (SEQ ID NO. 2); or

10 AAAAAAAAAAAAAAAAAAAGTATTACCGCACTCACTTGGACTTCTGTCAC
CGTCACCGCATAGCTCATCTACGTCTTCC (SEQ ID NO. 3), which may
optionally be hybridised to the sequence GGAAGACGTAGATGAGC (SEQ ID NO.
4)

The first and second adaptor sequences are preferably different to one another.

15

The first primer may have the nucleotide sequence:

GTATTACCGCACTCACTTGGGA (SEQ ID NO. 5).

The second primer may have the nucleotide sequence:

20 CATCGTCCTGGCGTCTGGCT (SEQ ID NO. 6).

The third primer may have the nucleotide sequence:

CTTCTGTCACCGTCACCGCATAG (SEQ ID NO. 7).

25 Preferably, the size exclusion agent is PEG.

The first restriction enzyme is preferably an enzyme having a recognition site an average of once per 250 nucleotides in a typical DNA sequence, most preferably *AluI*. Preferably, steps (e) and (f) of the method each continue for between 6 and 100 hours, more preferably between 30 and 50 hours, most preferably about 45 hours. In a further preference, step (g) of the method continues for about 24 hours. Preferably, the size exclusion agent (for example, PEG) is present in steps (e) and (f) at 4-8%, more preferably at about 6% and in step (g) at 15-20%, more preferably at about 17.5%.

30

The separation of DNA in step (h) may be carried out by any of the means well known to the skilled person, for example, by gel electrophoresis, band excision and purification, or using the marker molecule present on the first adaptor.

5

Brief Description Of The Figures

Embodiments of the invention will now be shown, by way of example only, with reference to the accompanying Figures 1-6 in which:

10 Figure 1 shows a schematic representation of the PCR based subtractive hybridisation;

Figure 2a shows normalisation and enrichment of cDNA fragments during the first hybridisation reaction.

15 Figure 2b shows the effect of polyethylene glycol on Hyg enrichment.

Figure 3 shows the effect of the amount of driver cDNA on the efficiency of subtractive hybridisation;

20 Figure 4 shows a schematic representation of the procedure to process DNA selected by subtractive hybridisation into an shRNA library using restriction sites on Adaptor A;

25 Figure 5 shows the difference in gene expression between a MEF cell line which grows on the surface of a culture dish cells or arrested in methyl cellulose; and

Figure 6 is a comparison of the abundance of differentially expressed genes as would be found in a completely normalised library (A), and in the shRNA library of the invention, as determined by sequencing (B); the figure shows the bins of differentially expressed genes, sequences which are not annotated as gene and genes which are not
30 expressed in the cell lines under study.

Examples*Oligonucleotides and cDNA*

All oligonucleotides were obtained from Sigma.

5 SH (subtractive hybridisation) Adaptor A

GTATTACCGCACTCACTTGGAGCATCGTCCTGGCGTCTGGCTGAGGGTAG
CTGGAGAGGCGATCCGACCG (SEQ ID NO. 1)

(Primer 1 recognition site is bold; primer 2 recognition site is italicised)

10 Antisense SH Adaptor A

CGGTCGGATCGC (SEQ ID NO. 2)

SH Adaptor B

BiotinAAAAAAAAAAAAAAAAAAAAAG**TATTACCGCACTCACTTGGACTTCT**

15 *GTCACCGTCACCGCATAGCTCATCTACGTCTTCC* (SEQ ID NO. 3)

(Primer 1 recognition site is bold; primer 3 recognition site is italicised)

Antisense SH Adaptor B

GGAAGACGTAGATGAGC (SEQ ID NO. 4)

20

SH Primer 1

GTATTACCGCACTCACTTGGGA (SEQ ID NO. 5)

SH Primer 2

25 BiotinCATCGTCCTGGCGTCTGGCT (SEQ ID NO. 6)

SH Primer 3

CTTCTGTCACCGTCACCGCATAG (SEQ ID NO. 7)

30 SH Primer 4

CATCGTCCTGGCGTCTGGC (SEQ ID NO. 9)

Looped Adaptor C

TTCAAGAGAACGCGTTGCACCGGTGCTGCACCGGTGCAGCGCGTTCTCTT
GAANN (where N A, G, C, or T) (SEQ ID NO. 8); or

TTCAAGAGAN₁N₂GCGTTGCACCGGTGCTGCACCGGTGCAGCGCn₂n₁TCTCT
TGAAN₃N₄ (where N₁, N₂, N₃, N₄, n₁ and n₂ are independently selected from A, G,
5 C, and T and n₁ is complementary to N₁ and n₂ complementary to N₂) (SEQ ID NO.
12);

To adapt the standard pRETRO Super for insertion of inserts produced by the method
of the invention, the following sequence is inserted between *Bgl*II and *Hind*III sites in
10 the pRetroSuper vector, to allow insertion of processed fragments after BsgI
digestion:

Adaptor for pRetroSuper (sense)

GATCCCCGCTGCCGTGCAGTTAACCTGCACCCGAGCTTTTTGGAAA (SEQ
15 ID NO. 10)

Adaptor for pRetroSuper (antisense)

AGCTTTTCCAAAAAGCTCGGGTGCAGGTAACTGCACGGCAGCGGG (SEQ
ID NO. 11)

20

cDNA synthesis tester and driver cDNA

RNA was isolated from cells using RNeasy columns (Qiagen). cDNA was produced
according to the SMART cDNA synthesis method (Clontech). cDNA was amplified
using the Expand polymerase (Roche) during a maximum of 20 PCR cycles in 45
25 separate 50µl reactions. After PCR, the cDNA was precipitated before purification on
a Microcon[®] YM100 column (Millipore).

Subtractive hybridisation

Adaptors A and B (Sigma) allow subtractive hybridisation and subsequent shRNA
30 library construction. Adaptor A contains sites for library processing and B is
biotinylated to allow pull down using beads. 1µg of tester cDNA preparation was *Alu*I
digested and ligated in 20µl buffer 4 (New England BioLabs), supplemented with
ATP (1mM), and either adaptor A or B (20µM). After incubation with *Alu*I (5U; 1

hour at 37°C), ligase (5U) was added to the reaction, which was incubated overnight in a thermocycler cycling between 30°C (10 sec) and 10°C (10 sec). After heat inactivation of the enzymes (20 min at 80°C), the samples were filtered twice to remove excess adaptors and concentrated (20ng/μl tester A and B) using a Microcon[®] YM 100 column.

2μg driver cDNA was digested overnight in 40 μl buffer 4 (NEB) using *AluI* (10U). After heat inactivation of the enzyme (20 min at 80°C), the sample was concentrated (20ng/μl tester A and B) and 200ng/μl driver) using a Microcon[®] YM 10 column.

For subtractive hybridisation, 10ng of both the adaptor A and B ligated tester fragments were each mixed with 700ng of driver fragments. In a volume of 5μl, containing PEG (6%; MW 6/8000), NaCl (0.5mM), HEPES (100mM), and covered by mineral oil, the fragments were melted using a thermocycler (3 min 95°C) and subsequently allowed to hybridise for 45 hours at 68°C. Then, the Adaptor A and B ligated samples, and 1.25μl PEG 55% were thoroughly mixed. Hybridisation was allowed to continue for 24 hours, before the reaction was diluted by the addition of 250μl of dilution buffer (50mM NaCl, HEPES 100mM) and kept at 68°C for 10 more minutes. The reaction was removed from the thermocycler and added to 100ng of streptavidin-coated magnetic beads (Dynalbeads) in 250μl dilution buffer. After 10 minutes at room temperature (while keeping the beads in suspension), the biotinylated Adaptor B ligated fragments were pulled down from the mixture on a magnet and washed twice with dilution buffer (300μl, 5 min, 68°C). The beads were resuspended in 100μl *pfu* polymerase mix (Invitrogen) containing only dNTPs (10mM each) and heated to 75°C before the *pfu* polymerase was added to hotstart the reaction and fill in the adaptor sequences (5 min). The reaction was stopped by the addition of EDTA and the beads were recovered on a magnet. After cooling, they were washed twice with NaOH (100nM) and once in tris buffer (10mM). After recovery on a magnet, half of the beads are used as a template in a subsequent nested PCR using the *pfu* polymerase.

In a nested PCR procedure, the first PCR reaction (1000μl *pfu* PCR mix divided over 40 separate 25μl reactions) used the primer 1 and consisted of 30 cycles (15 sec 94°C;

30 sec 64°C; 90 sec 94°C). After mixing of the duplicate reactions, 2µl was used as a template in the secondary PCR (1000 µl *pfu* PCR mix divided over 40 separate 25µl reactions) using primers 2 and 3 and consisting of 16 cycles (15 sec 94°C; 30 sec 64°C; 90 sec 94°C). Primer 1 was biotinylated for further applications. After PCR, the subtracted library was precipitated before purification on a Microcon® YM100 column.

Subtractive hybridisation using isogenic Hyg+ and Hyg- cell lines.

Enrichment of differentially expressed cDNA sequences for library construction is accomplished by PCR based subtractive hybridisation, as outlined above and in Figures 1 and 2.

mRNA sequences overexpressed in one transcriptome (tester) compared to another (driver) at a ratio of t/dr are selected. First, 5' ends (but not 3' ends) of *AluI* digested tester cDNA are ligated to either Adaptor A (step 1 of Figure 1) or B (step 2). Both Adaptor A and B ligated tester fragments are mixed separately with 75 fold excess *AluI* digested driver cDNA (step 3), melted and allowed to hybridise. After the first hybridisation, a normalised number (n) of each fragment remains single stranded. The share of remaining single stranded fragments which carry an adaptor is proportional to their abundance in the tester (t) and driver (dr) cDNA. The equalised number n of single strands present in the two samples is mixed and, after addition of PEG (step 4), new hybrids can be formed during the second hybridisation. The number of hybrids which carry two different adaptors at their ends is then proportional to their relative expression levels in the tester and driver cDNA. Subsequently, hybrids containing the adaptor B are fished out from the mixture (step 5) and, after addition of polymerase to fill in the complementary adaptor strand, can be exponentially amplified if they carry the adaptor A at their other end (step 6). Amplification of hybrids carrying two identical adaptors is suppressed due to hairpin formation which prevents primer annealing. Hybrids which carry a single adaptor are only amplified linearly.

Two Adaptors A and B were designed to allow subtractive hybridisation and subsequent processing of selected fragments into shRNA libraries. The Adaptor A contained several restriction sites to process attached sequences into an shRNA

library. The subtractive hybridisation procedure was optimised using a pair of isogenic embryonic stem cells differing in their hyg expression. In separate reactions, the Adaptors A and B were ligated to *AluI* digested cDNA obtained from cell line H⁺, which expresses the hyg gene (called tester cDNA). During a first hybridisation, the
5 Adaptor A and -B ligated samples were allowed to anneal separately to excess cDNA obtained from cell line H⁻, which lacks the hyg gene (driver cDNA).

Over time, a normalized number of each of the different *AluI* fragments present in the mixture is expected to remain single stranded due to the second order kinetics of
10 re-hybridisation, which causes the more abundant fragments to re-anneal faster until an equilibrium concentration is reached for each different fragment. In addition, for each of the different fragments, the proportion of remaining single strands carrying either one of the adaptors A or B depends on their relative abundance in the cell line H⁺ and H⁻ cDNA. Progressively more of the remaining single strands will carry an
15 adaptor when the expression of the sequence is reduced in cell line H⁻ relative to cell line H⁺.

After equilibrium was reached, the Adaptor A and B ligated samples were mixed; part of the remaining single strands were then forced to anneal during a second
20 hybridisation by increasing the polyethylene glycol concentration to raise their effective concentration. Because the strands were first equalised, a normalised number of hybrids were formed for each of the *AluI* fragments during the second hybridisation, including some hybrids which carry both an Adaptor A and B at their ends. Again, their number depended on the relative expression levels in the two cell
25 lines, and was strongly enriched for sequences which are under-represented in cell line H⁻. Therefore, the hybrids carrying two different adaptors represent putative target sequences desired to constitute the shRNA library, which are selected in two ways.

30 First, after immobilising adaptor B on magnetic beads, all of the adaptor A strands, which carry the restriction sites necessary to process attached cDNA sequences into shRNA libraries, were washed away unless they were hybridised to Adaptor B strand. Subsequently, to produce a subtracted library, only fragments carrying both adaptors

A and B at their ends were exponentially amplified by PCR, using nested primers located in the two different adaptors, further restricting the resulting shRNA library to the desired sequences.

5 *Optimisation of subtractive hybridisation procedure*

To optimise subtractive hybridisation, the time required to reach equilibrium for optimal normalisation during the first hybridisation was first determined.

10 Adaptor A- and B-ligated tester cDNA H⁺ and driver cDNA H⁻ were mixed 1:35, and the first hybridisation was allowed to proceed between 6 and 50 hours, before the two Adaptor A and B ligated samples were mixed and the PEG concentration was raised from 6% to 22%. After the second hybridisation (24 hour), the subtracted library was amplified by PCR, and the presence of a panel of 10 genes, including the differently expressed hyg gene and abundant genes (such as actin, tubulin, E cadherin, and msh6)
15 among the selected cDNA fragments was measured using dotblots. The results are shown in Figure 2a. When the first hybridisation was allowed to proceed for 45 hours, those genes expressed equally in the two cell lines H⁺ and H⁻ were reduced to normalised low levels in the subtracted library. Even the levels of the abundantly expressed sequences were effectively reduced. In contrast, the differentially expressed
20 hyg gene was strongly enriched and exceeded the other sequences approximately 200 fold in the subtracted library. The addition of PEG during the second hybridisation increases the efficiency of the subtractive hybridisation (Fig. 2b).

25 In a second experiment, the effect of the amount of driver cDNA added in excess on the efficiency of subtractive hybridisation was determined.

The procedure was followed using 15, 30, 45, or 60 fold excess of driver (H⁻) cDNA compared to tester (H⁺) cDNA for subtractive hybridisation. As expected, the efficiency of subtractive hybridisation was increased when more driver cDNA was
30 added to the reaction (Figure 3). When the first hybridisation was allowed to proceed for only 24 hours, normalisation and enrichment rapidly improved when increasing amounts of driver cDNA are added to the reaction.

Construction of shRNA libraries

Restriction sites on the Adaptor A were used to process the selected sequences into inverted repeats, which were ligated into a pRetroSuper vector. 10 µg of the amplified subtracted library was processed in 100µl buffer 4 (New England BioLabs), containing SAM (1mM) and the Looped Adaptor C. One hour after addition of *MmeI* (5U), ligase was added overnight to form hairpins by Looped Adaptor C ligation. After inactivation of these enzymes (80°C 15 min), *Nb.BbvC I* (5U) was added for 1 hour to nick the hairpin. On ice, streptavidin-coated magnetic beads (100ng) were used to pull down the biotinylated nicked hairpin. The supernatant was then replaced by 100 µl buffer 2 (New England BioLabs) containing primer 4 (100nM) and dNTP's (10mM each) the sample was heated (70°C 20 min) to inactivate the enzyme and release the nicked hairpins from the beads. *Exo⁻* Klenow polymerase (10U) is added to elongate the primer 4 (1 hour at 37°C) and form an inverted repeat. *BpmI* (10U) digestion (1 hour at 37°C) removed the duplicated adaptor A ends, generating CT overhangs on both sides. The sample was separated on a 4.5% agarose gel and the 91bp inverted repeat was isolated from gel by freeze squeezing and phenol extracted. After ligation into a modified pRetroSuper vector, the plasmids were used to transform bacteria. At least 40000 individual small colonies were pooled and a maxiprep (Genomed) was performed to isolate plasmids. 1µg was digested with *BsgI* to remove an obsolete part of the Looped Adaptor C, and the linearised 6834bp vector fragment isolated from gel (Qiagen). After religation of the vector, the plasmids were used to transform bacteria. The plasmids from 40000 individual small colonies were pooled and a maxiprep (Genomed) was performed. An aliquot of the maxiprep was used to transform *E. coli* in order to isolate and sequence individual plasmids. Sequenced inserts were compared to annotated sequences (Genbank). The library of pRETRO SUPER plasmids was calcium transfected into Phoenix cells to produce viruses.

The steps to process the selected cDNA sequences into an shRNA library are shown in Figure 4 and are outlined in Table 1:

1	<i>MmeI</i> digestion
2	Ligation with looped adaptor

3	Nicking by Nb.BbvCI enzyme
4	Immobilisation on beads and washing
5	Heating and replacement by primer
6	Replication, Klenow primed by nick
7	<i>BpmI</i> digestion
8	Separation and isolation from gel
9	Ligation into p(RETRO)Super
10	<i>BsgI</i> digestion and religation into p(RETRO)Super

Table 1: outline of experimental steps shown in Figure 4

Explaining now in more detail, digestion with *MmeI*, which cuts away from its recognition site in adaptor A, leaves around 18 base pairs of cDNA attached to the adaptor A. This sequence will constitute the sequence which is expressed in shRNA in the shRNA vector. The cDNA end is ligated to the looped adaptor C, forming a hairpin structure. The looped adaptor C carries a nine nucleotide sequence which ultimately ends up in between the inverted cDNA repeats in the final shRNA vector. In the hairpin consisting of adaptor A, cDNA and adaptor C, a nick is created in adaptor A using Nb.BbvCI. Since a biotinylated primer was used during PCR amplification of the subtracted library, the nicked hairpins can be immobilised on streptavidin coated beads,. Due to the nick, part of the hairpin is released from the bead by heating, leaving part of adaptor A attached to the bead. Primer 4, which binds to part of the exposed sequence of adaptor A, is added to restore the nicked hairpin. Using the nick as an entry site for DNA synthesis by the Klenow fragment of DNA polymerase I, a complementary strand is synthesized, starting from primer 4, to form a double stranded inverted repeat. *BpmI*, which cuts 16 bp away from its restriction recognition site present in adaptor A, is used to remove the now duplicated adaptor A ends. The resulting inverted repeats of cDNA and adaptor C sequence are purified by gel electrophoresis. Subsequently, they are ligated behind an appropriate promoter sequence, preferably the H1 promoter, in a vector of choice, preferably pRETRO-SUPER. Finally, most of the adaptor C sequence is removed using *BsgI*, which recognizes two restriction sites in adaptor C, to yield a pRETRO-Super shRNA vector. In the vector, inverted cDNA sequences are separated by the nine nucleotide sequence originally present in adaptor C.

The skilled person would readily be able to determine suitable alternative adaptors, primers, vectors and/or restriction enzymes for use in the method of the invention.

5 An shRNA library was produced using a subtracted library generated from cell line H+ and H- using 60 fold excess of cell line H- as a driver. To validate the resulting shRNA library, the inserted sequences in 88 shRNA plasmids were verified by sequencing. 81 of the sequences corresponded to inverted repeats capable of expressing genuine shRNA molecules. The inserted sequences matched perfectly to
10 mRNA sequences found in GenBank. They all followed *AluI* sites and were distributed over the full length of the corresponding mRNA. It was found that one of the 88 vectors targeted the hyg gene, the result of efficient enrichment by subtractive hybridization.

15 *Validation of subtracted libraries.*

The composition of the subtracted libraries was determined by microarray, dotblot analysis or sequencing. Microarrays were performed by the Netherlands Cancer Institute microarray facility. For dotblots, a panel of 15 genes was spotted onto a membrane and hybridised with radiolabelled subtracted libraries. The abundance of
20 the panel genes in the libraries was quantified by phosphoimaging (Figures 2 and 3). Sequencing was performed at the Netherlands Cancer Institute sequencing facility.

Production of shRNA library from cells either grown on a culture dish or arrested in methyl cellulose.

25 To test the method of the invention, an shRNA library was produced, which was enriched for tumour suppressor sequences that are induced when cells arrest in methyl cellulose. Subsequently, a loss of function screen for colony growth in methyl cellulose was performed. For microarray analysis and library construction, cDNA was produced from a mouse embryonic fibroblast (MEF) cell line expressing RAS^{V12} and
30 lacking the retinoblastoma proteins Rb and p107, which was either grown on a culture dish or arrested in methyl cellulose to induce tumor suppressor genes. Subtractive hybridization was performed using cDNA derived from the arrested cells as the tester

and from the growing cells as a driver to enrich for gene sequences induced by methyl cellulose.

Microarray analysis of cells residing in methyl cellulose revealed numerous differences in gene expression, including many genes which were induced in the arrested cells, but not in the growing cells. Figure 5 shows the expression profile of the growing and arrested cells as determined by microarray analysis. Black dots represent genes expressed equally in the two culture situations. Grey dots represent genes either overexpressed (negative M value) or repressed (positive M value) by culturing the cells in methyl cellulose. The 21331 expressed genes were divided into 5 bins: more than 4-fold induction (47 genes), 4-3 fold (83 genes), 3-2 fold (425 genes), 1-2 fold (10490 genes) or reduced expression (10440 genes). In Figure 6, the representation of the different bins as would be found in a normalised library containing all 25000 annotated genes (i.e., genes that encode a protein) (A) is compared to the representation of the bins in the library made according to the invention (B). The genes induced mostly by methyl cellulose (>4-fold induction) were enriched up to 51 fold.

Next, untransformed RAS^{V12} expressing Rb/p107 -/- cells either mock infected or infected with the shRNA library of the invention were inoculated in methyl cellulose. Although RAS^{V12} expressing Rb/p107 -/- cells normally arrest under these conditions, expression of the library of the invention caused a small number of colonies to form, indicating that this system can be used to screen for tumor suppressor sequences. Sequencing of vectors indicated that they target both coding and non-coding genes (Table 2).

Table 2

Target	Frequency	Function	Reference
MAPKAP K3	12	p38 pathway, regulation BMI1 and p19	(Voncken, Niessen et al. 2005)
Hspb1	1	p38 pathway, growth suppression and invasion	(Xu, Chen et al. 2006; Matsushima-Nishiwaki, Takai et al. 2008)
TNF α induced protein	1		
Dishevelled 3	1	Wnt signaling	(Polakis 2000)
Sca1 (Ly6a, ataxin)	2	Stem cell factor regulating proliferation and differentiation	(Epling, King et al. 2008)
Dppa5	2	Stem cell factor implicated in pluripotency	(Kim, Suh et al. 2005)
Ptrf (cavin)	1	Formation caveoli through regulation tumor suppressor caveolin-1	(Williams, Hassan et al. 2005; Williams and Lisanti 2005; Hill, Bastiani et al. 2008)
Enam/EIF-4G 3	6	unknown	
Non coding sequence on chromosome 7	3	unknown	
Non coding sequence on chromosome 8	1	unknown	

Conclusions

Expression profiling using microarrays may reveal many genes which are differentially expressed between two different cellular phenotypes. However, it usually fails to identify the genes responsible to bring about this phenotypic change, because it is impossible to distinguish between cause and effect. To identify the genes responsible for a phenotypic transition, functional genetic screens may be performed. In recent years, loss of function screening using viral RNAi libraries has proved to be a powerful method to study gene function. Loss of function screens are facilitated by reducing the complexity of the RNAi library. Since fewer cells require testing to cover the variety of library sequences, this benefits the ratio between truly positive “hits” and obligatory background. In addition, it becomes easier to perform screens in a single well format. Finally, when the complexity of the library becomes low enough, it may be possible to pick up interactions between genes in screens.

To reduce their complexity, RNAi libraries for loss-of-function screening should ideally be limited to genes which are suspected to suppress the particular selectable phenotype which is the focus of the screen, such as the genes which are actually differentially expressed during the phenotypic change.

Although post-translational modifications of proteins may play an important role to bring about a phenotypic change, it may be beneficial to use shRNA libraries as

disclosed here, if there are accompanying changes in expression patterns, for two reasons. First, the subpopulation of genes which are regulated during a phenotypic transition are strongly suspected to play a underlying role. At the expense of disregarding others, these candidate genes are strongly enriched to lower the complexity of libraries of the invention. Second and equally important, because the sequences selected in the library were regulated during the phenotypical transition, they actually play a role in the physiological context of cells.

Using the method of the invention, more shRNA vectors can be generated per gene compared to conventional synthetic shRNA libraries, depending on the number of *AluI* sites present in a gene, which averages 20. This provides an internal control for off-target effects and increases the chance to come across vectors that give the right degree of functionality. In addition, libraries may be produced for every organism. In addition to pRetroSuper, other shRNA delivering vector backbones, including retroviral or lentiviral vectors, vectors encoding inducible RNAi or microRNA primary transcripts, can be used with only a minor adaptation to meet experimental demands.

An important advantage is the simplicity of the method. After isolation of RNA and the production of cDNA, it requires only three weeks to perform the subtractive hybridisation and to produce tailor-made libraries.

Claims

1. A method of preparing a shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and second cell type, the method comprising the steps of: pre-identifying said one or more cDNA sequences using a first adaptor DNA sequence; and processing said one or more cDNA sequences using the first adaptor DNA sequence.
2. A method according to claim 1 wherein the first and second cell types are phenotypically different from one another.
3. A method according to claim 1 or 2 wherein the first and second cell types are different cell strains.
4. A method according to claim 1 or 2 wherein the first and second cell types are the same cell strain.
5. A method according to any preceding claim, wherein the step of pre-identifying said one or more cDNA sequences comprises using subtractive hybridisation using the first adaptor DNA sequence and a second adaptor DNA sequence.
6. A method according to any preceding claim, wherein said one or more cDNA sequences are processed to produce inverted repeat sequences encoding for shRNA molecules directed to the at least one gene which is differently expressed in the first and second cell type.
7. A method according to any preceding claim, wherein said one or more cDNA sequences are processed using: the first adaptor DNA sequence which comprises a recognition site for a first restriction enzyme; and a third adaptor DNA sequence to form hairpin structures.

8. A method according to any one of preceding claim wherein the step of pre-identifying each one or more cDNA sequences comprises the steps of:
- a. carrying out a restriction digest reaction of cDNA from the first cell type using a first restriction enzyme;
 - b. ligating a first adaptor DNA sequence, comprising a first primer recognition sequence and a second primer recognition sequence and a marker molecule, to DNA from step (a);
 - c. ligating a second adaptor DNA sequence comprising a first primer recognition sequence and a third primer recognition sequence to DNA from step (a);
 - d. carrying out a restriction digest reaction of cDNA from the second cell type using the first restriction enzyme;
 - e. mixing the denatured products of step (b) with denatured products of step (d), optionally in the presence of a size exclusion agent, in an annealing step;
 - f. mixing the denatured products of step (c) with denatured products of step (d), optionally in the presence of a size exclusion agent, in an annealing step;
 - g. mixing the denatured products of steps (e) and (f) in the presence of a size exclusion agent in an annealing step, wherein the concentration of the size exclusion agent is higher than the concentration of size exclusion agent in steps (e) and (f), if present;
 - h. separating and isolating DNA carrying the first adaptor sequence from DNA carrying only the second adaptor sequence or no adaptor sequence, or separating and isolating DNA carrying the second adaptor sequence from DNA carrying only the first adaptor sequence or no adaptor sequence;
 - i. carrying out a nested PCR reaction using the separated DNA from (h) as template DNA and including the use of a first primer which recognises the first primer recognition sequence and, subsequently, a second primer which recognises the second primer recognition sequence and a third primer which recognises the third primer recognition sequence.
9. A method according to claim 8 wherein the marker molecule is biotin or streptavidin.
10. A method according to claim 8 or 9 comprising the further step of inserting at least a portion of the PCR product from (i) into an shRNA expression vector.

11. A method according to any one of claims 1 to 9, wherein the processing step comprises the steps of:
- digesting the one or more cDNA sequences with a first restriction enzyme to produce a fragment of each of said one or more cDNA sequences attached to the first adaptor sequence;
 - ligating the product of (a) with the third hairpin adaptor DNA sequence to form hairpin structures;
 - replicating the hairpin structures of step (b) to form an inverted repeat of each of said one or more cDNA sequences;
 - removing at least a portion of the duplicated first adaptor DNA sequences from the product of step (c) using a second restriction enzyme; and
 - processing the product of step (d), if necessary, to encode for a shRNA.
12. A method according to any one of claims 1 to 9, wherein the processing step comprises the steps of:
- carrying out a restriction digest reaction of the one or more pre-identified cDNA sequences using a second restriction enzyme;
 - ligating the product of (a) with a third adaptor sequence, comprising a recognition site for a third restriction enzyme, to form hairpin structures;
 - nicking the hairpin structure of step (b) using a third restriction enzyme;
 - isolating the product of the reaction in step (c) by binding to the marker molecule;
 - heating the product isolated in (d) with a fourth primer;
 - extension of the fourth primer by a polymerase to generate the inverted DNA sequence complementary to the product of (e); and
 - isolating at least a portion of the generated DNA product by use of a fourth restriction enzyme and inserting the resulting DNA into an shRNA expression vector.
13. A first adaptor nucleotide sequence for use in preparing a shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and second cell type, the adaptor sequence comprising:

a first primer binding site for PCR amplification of a cDNA attached thereto;
a first restriction recognition site for a first restriction enzyme to cause the cleavage of the cDNA attached to the first adaptor sequence into a fragment; and
a second restriction recognition site for a second restriction enzyme to cause the cleavage of the cDNA fragment from at least a portion of the first adaptor sequence.

14. The adaptor sequence of claim 13, wherein the first restriction recognition site for a first restriction enzyme causes the cleavage of the cDNA attached to the first adaptor sequence into a fragment of between about 10 and about 30 nucleotides in length.
15. The adaptor sequence of claim 13 or claim 14, further comprising a third restriction recognition site for a third restriction enzyme to introduce a nick into the first adaptor sequence.
16. The adaptor sequence of any one of claims 13 to 15, further comprising a third primer binding site for binding a primer to allow replication of the cDNA.
17. A kit for preparing an shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and second cell type, the kit comprising a first adaptor sequence according to any one of claims 13 to 16.
18. The kit of claim 17, further comprising a second adaptor sequence for use in subtractive hybridisation.
19. The kit of claim 17 or claim 18, further comprising a third hairpin adaptor sequence.
20. An shRNA library enriched for one or more cDNA sequences corresponding to at least a portion of at least one gene which is differently expressed in a first and a second cell type, wherein the shRNA library is obtainable by the method of any of claims 1 to 12.

21. A method of determining the effects of reduction in expression of a gene of interest, comprising the use of an shRNA library according to claim 20.

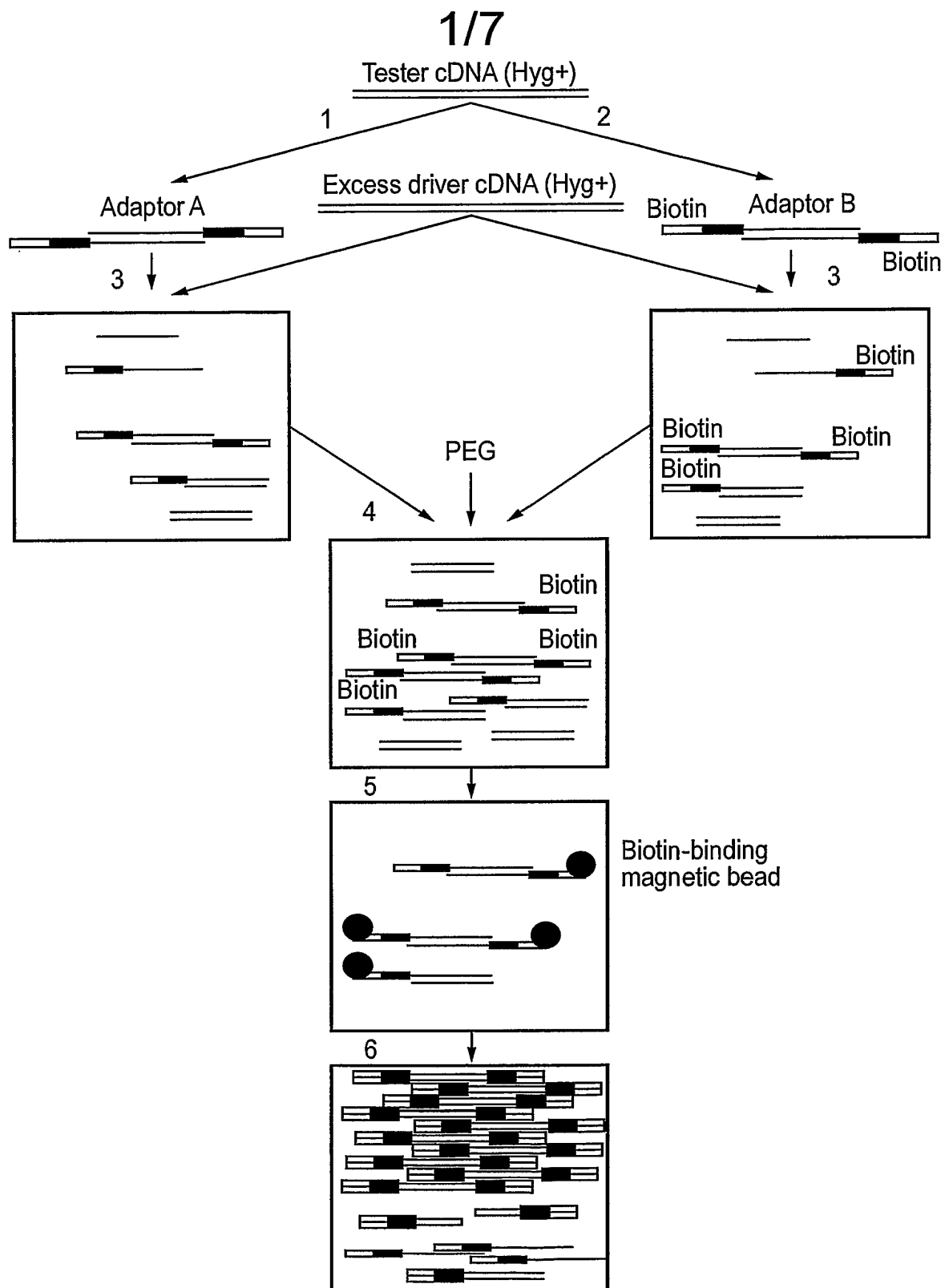


FIG. 1

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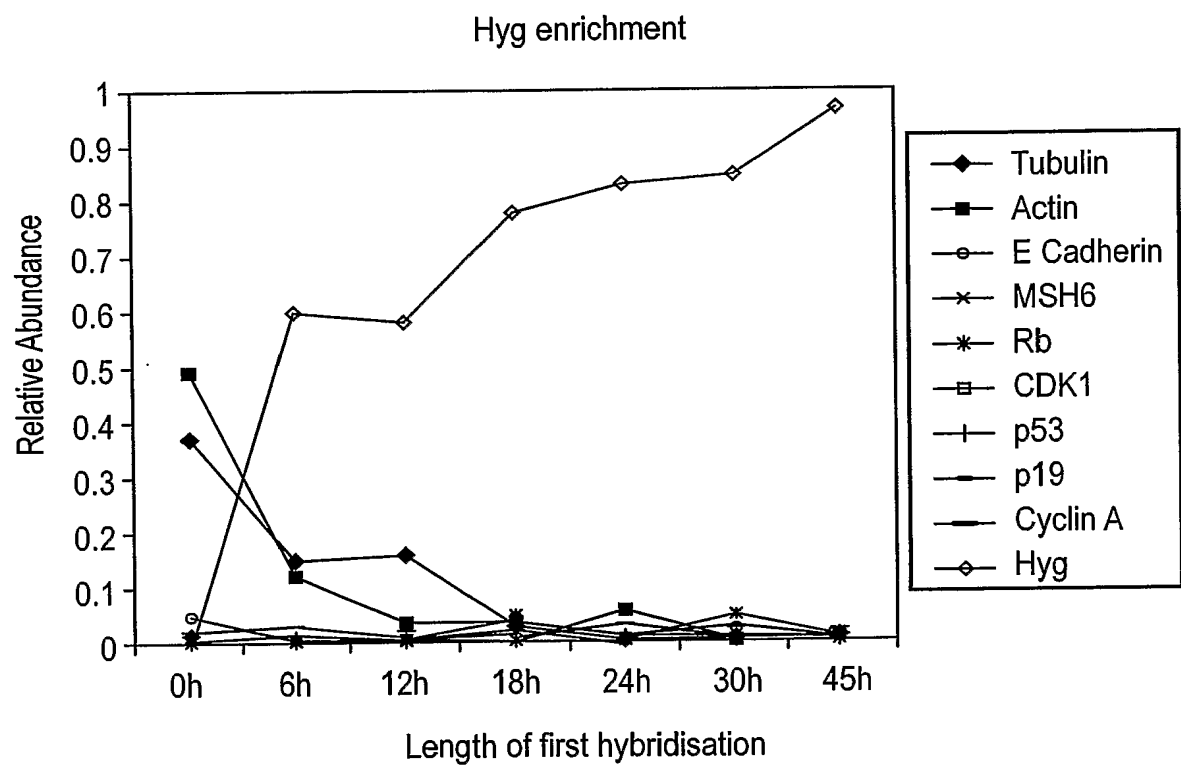


FIG. 2A

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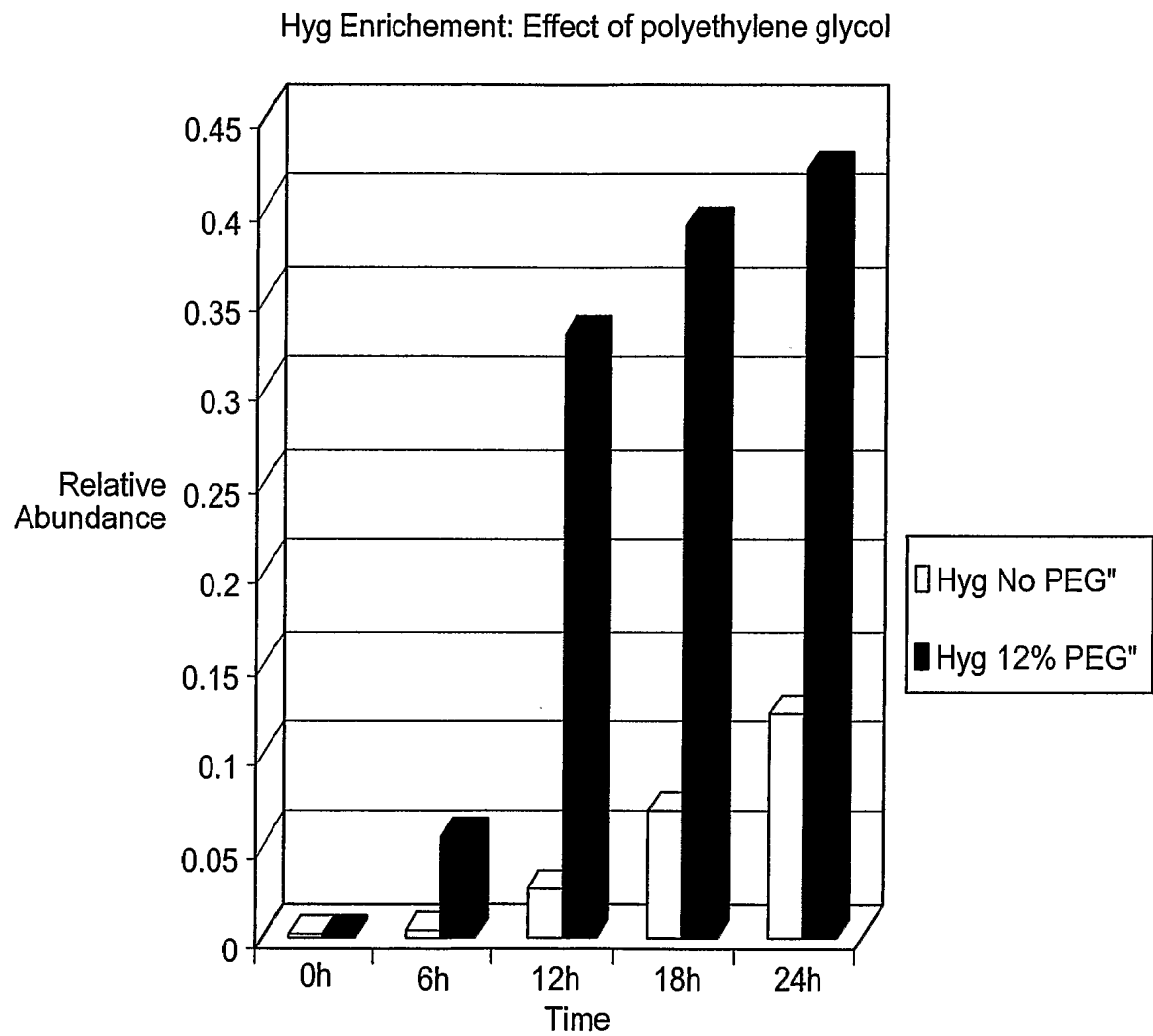


FIG. 2B

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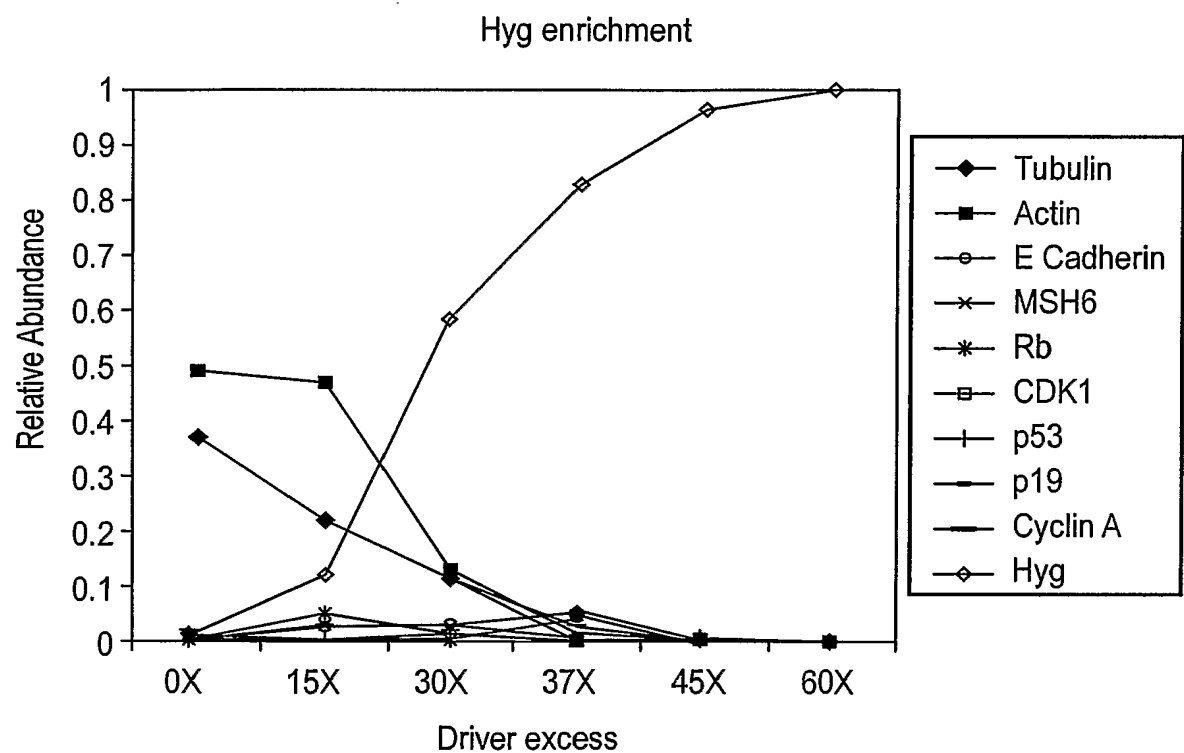


FIG. 3

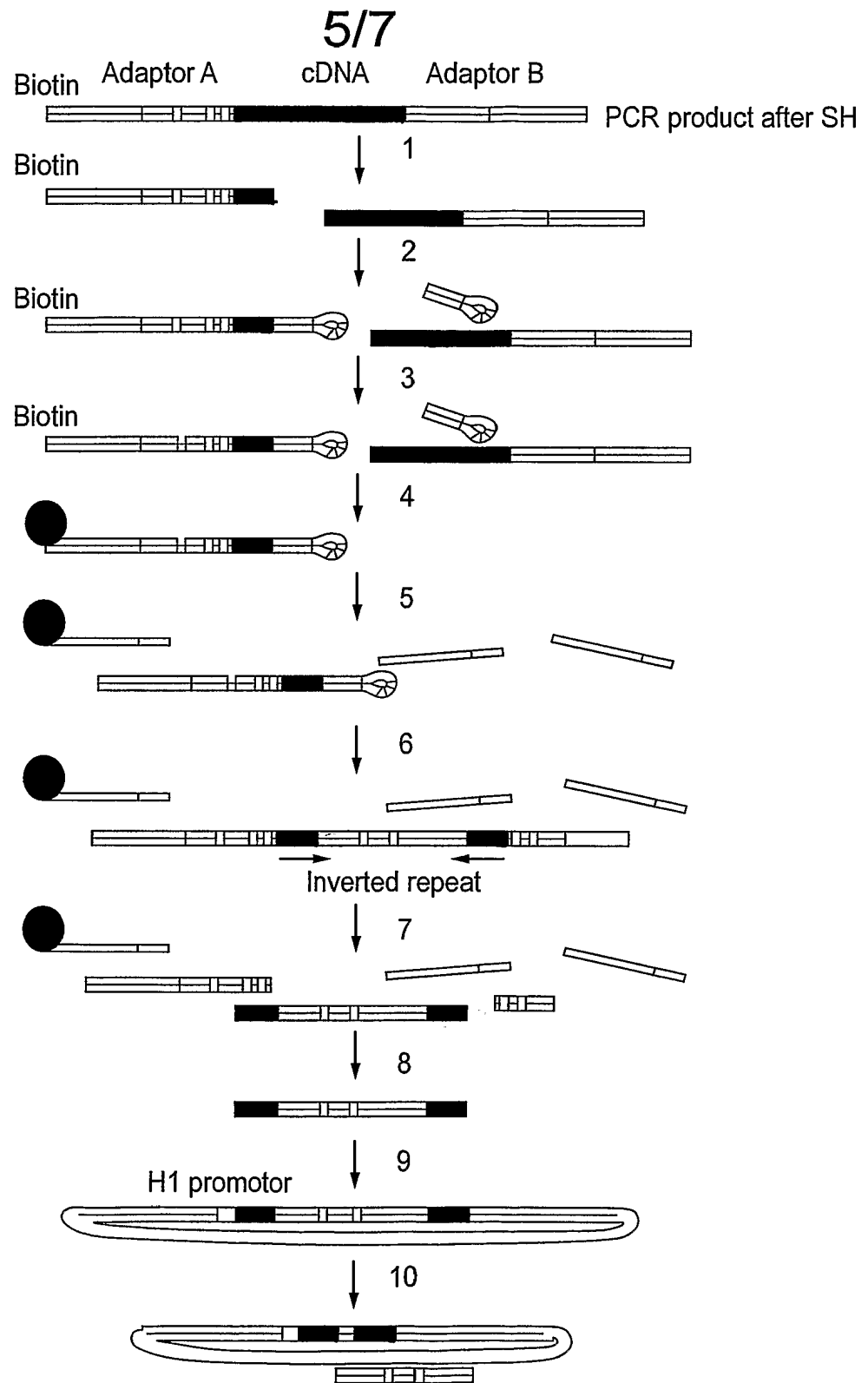


FIG. 4

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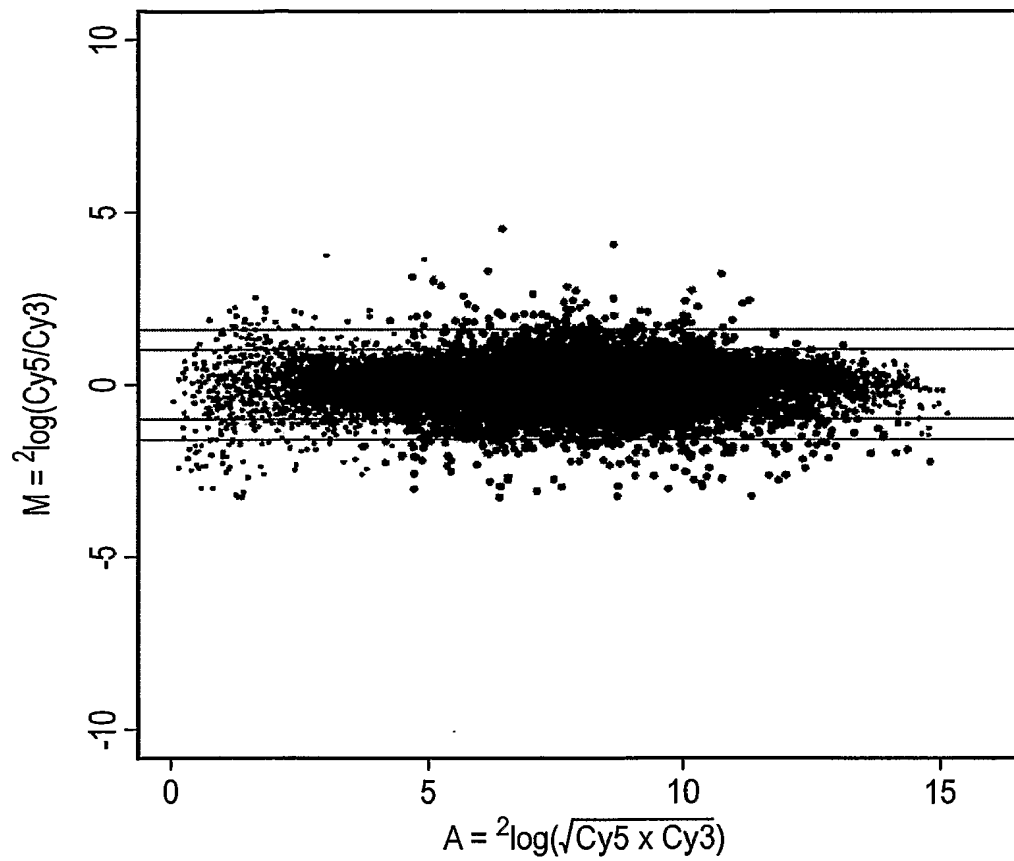


FIG. 5

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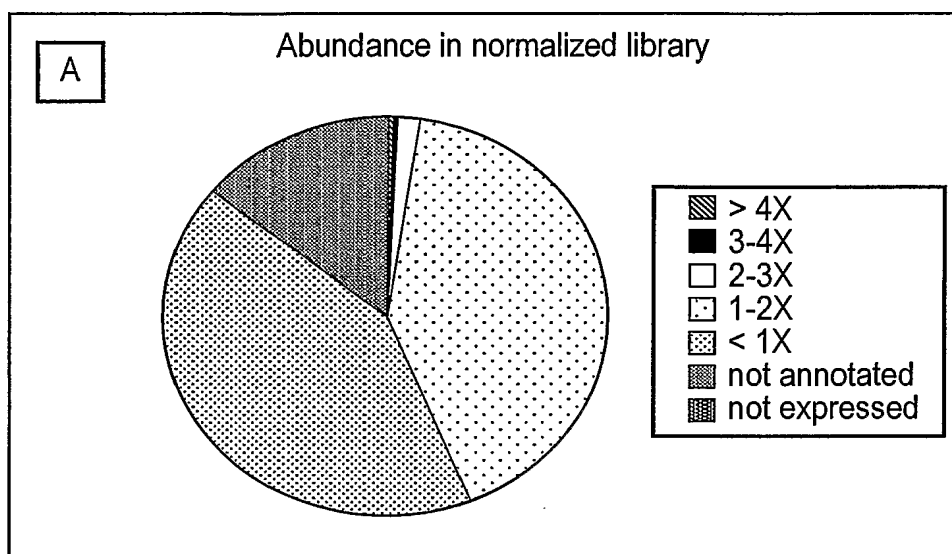


FIG. 6A

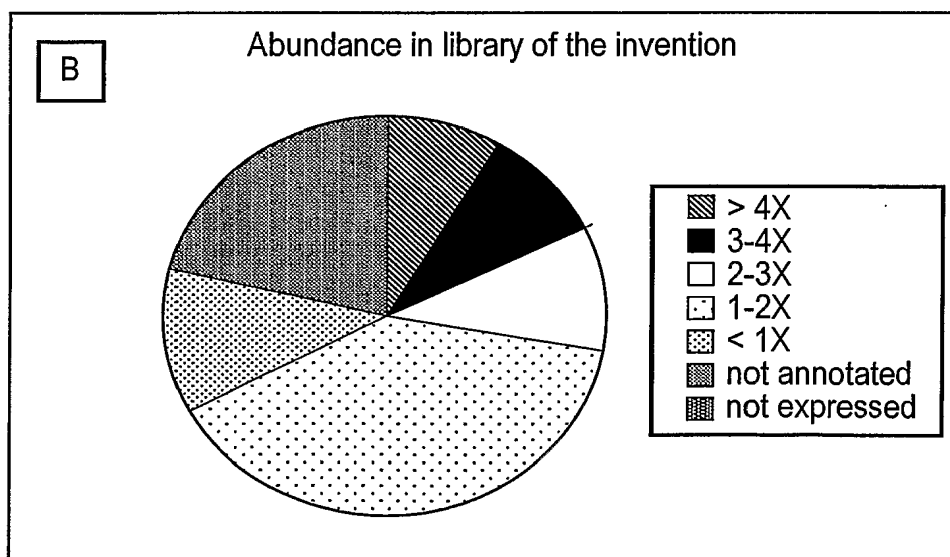


FIG. 6B

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/000684

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N15/10 C12N15/11		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XU ET AL: "Construction of equalized short hairpin RNA library from human brain cDNA" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 128, no. 3, 24 January 2007 (2007-01-24), pages 477-485, XP005856745 ISSN: 0168-1656 cited in the application the whole document ----- -/--	1-21
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
<p>* Special categories of cited documents :</p> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*&* document member of the same patent family</p>		
Date of the actual completion of the international search 24 June 2009		Date of mailing of the international search report 14/07/2009
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Ferreira, Roger

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2009/000684

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>ZHANG JUN-ZHENG ET AL: "Screening for genes essential for mouse embryonic stem cell self-renewal using a subtractive RNA interference library." STEM CELLS (DAYTON, OHIO) DEC 2006, vol. 24, no. 12, December 2006 (2006-12), pages 2661-2668, XP002533583 ISSN: 1066-5099 cited in the application the whole document</p>	1-21
X	<p>WO 2005/023991 A (GEN HOSPITAL CORP [US]; MCCAMPBELL ALEX [US]; KAZANTSEV ALEKSEY G [US]) 17 March 2005 (2005-03-17) abstract page 2, line 27 - page 9, line 32 page 12, line 27 - page 17, line 34 page 24, line 7 - line 34 claims 1-54 figures 1-4 examples 1-8</p>	1-21
X	<p>WO 03/033673 A (AGY THERAPEUTICS INC [US]; MELCHER THORSTEN [US]; MCFARLAND K C [US];) 24 April 2003 (2003-04-24) abstract page 2, line 1 - page 7, line 8 page 18, line 1 - page 22, line 11 page 31, line 5 - page 36, line 22 claims 1-51 figures 1-10 examples 1-7</p>	1-21
A	<p>HANAZAWA M ET AL: "Use of cDNA subtraction and RNA interference screens in combination reveals genes required for germ-line development in Caenorhabditis elegans." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 17 JUL 2001, vol. 98, no. 15, 17 July 2001 (2001-07-17), pages 8686-8691, XP002533584 ISSN: 0027-8424 the whole document</p>	1-21

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/000684

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	<p>ZHUMABAYEVA B ET AL: "Generation of full-length cDNA libraries enriched for differentially expressed genes for functional genomics." BIOTECHNIQUES MAR 2001, vol. 30, no. 3, March 2001 (2001-03), pages 512-516, 518, XP002533586 ISSN: 0736-6205 the whole document</p>	1-21
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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/000684

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DIATCHENKO L ET AL: "SUPPRESSION SUBTRACTIVE HYBRIDIZATION: A METHOD FOR GENERATING DIFFERENTIALLY REGULATED OR TISSUE-SPECIFIC CDNA PROBES AND LIBRARIES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC.; US, vol. 93, 1 June 1996 (1996-06-01), pages 6025-6030, XP002911922 ISSN: 0027-8424 cited in the application the whole document</p>	1-21
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P,X	<p>ZHOU DEMIN ET AL: "Generation of shRNA pool library: a revision of the biological technique from the viewpoint of chemistry." CHEMBIOCHEM : A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY 16 JUN 2008, vol. 9, no. 9, 16 June 2008 (2008-06-16), pages 1365-1367, XP002533588 ISSN: 1439-7633 the whole document</p>	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2009/000684

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 2005023991	A	17-03-2005	NONE	
WO 03033673	A	24-04-2003	CA	24-04-2003
			EP	11-08-2004
			2461171 A1	
			1444362 A2	

Form PCT/ISA/210 (patent family annex) (April 2005)

Electronic Acknowledgement Receipt

EFS ID:	38363176
Application Number:	16017340
International Application Number:	
Confirmation Number:	2635
Title of Invention:	COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION
First Named Inventor/Applicant Name:	Doug AMORESE
Customer Number:	21710
Filer:	Thomas C. Meyers/Michelle Aiello
Filer Authorized By:	Thomas C. Meyers
Attorney Docket Number:	NUGN-037/04US 34079/440
Receipt Date:	22-JAN-2020
Filing Date:	25-JUN-2018
Time Stamp:	12:26:30
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	NUGN-037-04US-IDS.pdf	1035748 b27fd2ab539122eda59abde5bf93f3eeb68ad92f	no	6

Warnings:

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2	Foreign Reference	WO2009112844A1.pdf	2638836	no	58
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5	Foreign Reference	WO2013112923A1.pdf	3568013	no	65
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6	Non Patent Literature	34079_440US_-_Borodina-2011-MethEnzymol.pdf	228021	no	20
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8	Non Patent Literature	34079_440US_-_EP-13740653-Search-Rpt-072315.pdf	344717	no	9
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9	Non Patent Literature	34079_440US_-_ISRWO-PCTUS2013023278-012513.pdf	865925	no	11
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10	Non Patent Literature	34079_440US_-_Levin-2010-NatMethods.pdf	2338709	no	18
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11	Non Patent Literature	34079_440US_-_Tewhey-2009-NatBiotech.pdf	849248	no	24
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12	Non Patent Literature	34079_440US_-_Tucker-2009-AmJHumGenet.pdf	1100925	no	13
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Warnings:					
Information:					
Total Files Size (in bytes):			21631403		

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National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

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Espacenet

Bibliographic data: WO2011156529 (A2) — 2011-12-15**METHODS AND COMPOSITION FOR MULTIPLEX SEQUENCING**

Inventor(s): RAYMOND CHRISTOPHER [US]; KURN NURITH [US]; MAGNUS JILL [US] ± (RAYMOND, CHRISTOPHER, ; KURN, NURITH, ; MAGNUS, JILL)

Applicant(s): NUGEN TECHNOLOGIES INC [US]; RAYMOND CHRISTOPHER [US]; KURN NURITH [US]; MAGNUS JILL [US] ± (NUGEN TECHNOLOGIES, INC, ; RAYMOND, CHRISTOPHER, ; KURN, NURITH, ; MAGNUS, JILL)

Classification: - **international:** **C07H21/02; C40B30/00; C40B30/04; C40B40/06**
- **cooperative:** **C12Q1/6855; C12Q1/6869** [more](#)

Application number: WO2011US39683 20110608 [Global Dossier](#)

Priority number(s): [US20100352801P 20100608](#)

Also published as: [WO2011156529 \(A3\)](#) [CN103119439 \(A\)](#) [EP2580378 \(A2\)](#)
[EP2580378 \(A4\)](#) [US2011319290 \(A1\)](#)

Abstract of WO2011156529 (A2)

Adapters are joined to target polynucleotides to create adapter-tagged polynucleotides. Adapter- tagged polynucleotides are sequenced simultaneously and sample sources are identified on the basis of barcode sequences.

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
15 December 2011 (15.12.2011)

(10) International Publication Number
WO 2011/156529 A2

PCT

(51) International Patent Classification:

C40B 40/06 (2006.01) **C40B 30/04** (2006.01)
C40B 30/00 (2006.01) **C07H 21/02** (2006.01)

(21) International Application Number:

PCT/US2011/039683

(22) International Filing Date:

8 June 2011 (08.06.2011)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/352,801 8 June 2010 (08.06.2010) US

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(57) Abstract: Adapters are joined to target polynucleotides to create adapter-tagged polynucleotides. Adapter-tagged polynucleotides are sequenced simultaneously and sample sources are identified on the basis of barcode sequences.



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METHODS AND COMPOSITIONS FOR MULTIPLEX SEQUENCING

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 61/352,801, filed June 8, 2010, which application is incorporated herein by reference.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on June 8, 2011, is named 25115-741-201.txt and is 21 Kilobytes in size.

BACKGROUND OF THE INVENTION

[0003] Large-scale sequence analysis of DNA can provide understanding of a wide range of biological phenomena related to states of health and disease, both in humans and in many economically important plants and animals, see e.g. Collins et al (2003), *Nature*, 422: 835-847; Service, *Science*, 311: 1544-1546 (2006); Hirschhorn et al (2005), *Nature Reviews Genetics*, 6: 95-108; National Cancer Institute, Report of Working Group on Biomedical Technology, "Recommendation for a Human Cancer Genome Project," (February, 2005); Tringe et al (2005), *Nature Reviews Genetics*, 6: 805-814. The need for low-cost high-throughput sequencing and re-sequencing has led to the development of several new approaches that employ parallel analysis of many target DNA fragments simultaneously, e.g. Margulies et al, *Nature*, 437: 376-380 (2005); Shendure et al (2005), *Science*, 309: 1728-1732; Metzker (2005), *Genome Research*, 15: 1767-1776; Shendure et al (2004), *Nature Reviews Genetics*, 5: 335-344; Lapidus et al, U.S. patent publication US 2006/0024711; Drmanac et al, U.S. patent publication US 2005/0191656; Brenner et al, *Nature Biotechnology*, 18: 630-634 (2000); and the like. Such approaches reflect a variety of solutions for increasing target polynucleotide density and for obtaining increasing amounts of sequence information within each cycle of a particular sequence detection chemistry.

[0004] Given the complexity of the mixture of sequences in a given reactions, sequencing is typically restricted to one sample per reaction chamber. However, the number of bases read in a given reaction using these next generation sequencing technologies can be far greater than that actually needed to acquire the sequence information of interest, which essentially amounts to wasted sequencing space. Coupled with increasing desires to sequence samples from multiple sources, the expense of utilizing these technologies can quickly become prohibitive. Sequencing runs are also often limited in the number of separate reactions that can be run in parallel, which places further restrictions on the efficiency with which large numbers of samples can be processed.

[0005] Some approaches to resolve these challenges have involved the incorporation of additional identifier sequences into each of the target fragments analyzed. Where different sequences are used for different samples, sequencing of pooled samples can be followed by resolution of sequences into subsets corresponding to sample sources based on the added sequences. However, addition of sequences to

resolve sample sources faces two challenges. Firstly, random errors in sequencing can make it impossible to correctly identify an appended identifier sequence with its sample source when such errors occur within appended sequences that are either too short or insufficiently dissimilar from sequences corresponding to other samples. Secondly, the addition of longer sequences to allow for such sequencing error takes up valuable sequencing space from target reads that can be as short as 20 bases. In view of these limitations, there is a need to increase the efficiency of next generation sequencing technologies such that samples can be sequenced in greater numbers, with greater identification accuracy, while maximizing the available sequencing space.

SUMMARY OF THE INVENTION

[0006] In one aspect, the invention provides methods, compositions, and kits for multiplex sequencing. In one embodiment, the method comprises sequencing a plurality of target polynucleotides in a single reaction chamber, wherein said target polynucleotides are from two or more different samples; and identifying the sample from which each of said sequenced target polynucleotides is derived with an accuracy of at least 95% based on a single barcode contained in the sequence of said target polynucleotide. In some embodiments, the target polynucleotides comprise one or more sequences with which the sequencing reaction is calibrated. In some embodiments, each barcode differs from every other barcode at at least three nucleotide positions. In some embodiments, the identification of sample source is accurate after the mutation or deletion of a nucleotide in the barcode.

[0007] In another aspect, the invention provides methods, compositions, and kits for producing adapter-tagged target polynucleotides from a plurality of independent samples. In one embodiment, the method comprises: (a) providing a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions; and (b) joining at least one of said first adapter oligonucleotides to said target polynucleotides of each of said samples, such that no barcode sequence is joined to said target polynucleotides of more than one of said samples. In some embodiments, the method further comprises (c) joining at least one of a plurality of second adapter oligonucleotides to said target polynucleotides of each of said samples from step (b), such that at least some of said target polynucleotides comprise said first adapter oligonucleotide at one end and said second adapter oligonucleotide at the other end. One or more of the adapter oligonucleotides of the present invention can comprise SEQ ID NO: 1. One or more of the adapter oligonucleotides of the present invention can comprise SEQ ID NO: 2. One or more of the adapter oligonucleotides can comprise a hairpin structure. One or more of the adapter oligonucleotides can comprise an oligonucleotide duplex.

[0008] In some embodiments, the barcode sequences are at least 3 nucleotides in length. In some embodiments, the plurality of barcode sequences includes sequences selected from the group consisting of: AAA, TTT, CCC, and GGG. In some embodiments, the plurality of barcode sequences includes sequences selected from the group consisting of: AAAA, CTGC, GCTG, TGCT, ACCC, CGTA, GAGT,

TTAG, AGGG, CCAT, GTCA, TATC, ATTT, CACG, GGAC, and TCGA. In some embodiments, the plurality of barcode sequences includes sequences selected from the group consisting of: AAAAA, AACCC, AAGGG, AATTT, ACACG, ACCAT, ACGTA, ACTGC, AGAGT, AGCTG, AGGAC, AGTCA, ATATC, ATCGA, ATGCT, ATTAG, CAACT, CACAG, CAGTC, CATGA, CCAAC, CCCCA, CCGGT, CCTTG, CGATA, CGCGC, CGGCG, CGTAT, CTAGG, CTCTT, CTGAA, CTTCC, GAAGC, GACTA, GAGAT, GATCG, GCATT, GCCGG, GCGCC, GCTAA, GGAAG, GGCCT, GGGGA, GGTTC, GTACA, GTCAC, GTGTG, GTTTT, TAATG, TACGT, TAGCA, TATAC, TCAGA, TCCTC, TCGAG, TCTCT, TGACC, TGCAA, TGGTT, TGTGG, TTAAT, TTCCG, TTGGC, and TTTTA.

[0009] In some embodiments, the method further comprises pooling the target polynucleotides from step (c). Target polynucleotides can be pooled based on the barcode sequences to which they are joined, such that all four bases are evenly represented at one or more positions along each barcode in the pool.

[0010] In some embodiments, target polynucleotides comprise fragmented sample polynucleotides. Fragmentation can comprise subjecting sample polynucleotides to acoustic sonication, and/or treating sample polynucleotides with one or more enzymes under conditions suitable for the one or more enzymes to generate random double-stranded nucleic acid breaks (which can include DNase I, Fragmentase, and variants thereof). In some embodiments, fragmentation comprises treating the sample polynucleotides with one or more restriction endonucleases. Fragments can have an average length of 10 to 10,000 nucleotides, such as an average length of 100-2,500 nucleotides, or 50-500 nucleotides. In some embodiments, samples comprise less than 500ng of nucleic acid. Target polynucleotides can comprise genomic DNA, DNA produced by a primer extension reaction, cDNA, mitochondrial DNA, chloroplast DNA, plasmid DNA, bacterial artificial chromosomes, yeast artificial chromosomes, or a combination thereof.

[0011] In some embodiments, the method further comprises performing the step of extending one or more 3' ends of the target polynucleotides, using the one or more joined adapter oligonucleotides as template. In some embodiments, the method further comprises amplifying the target polynucleotides after the extending step using a first primer and a second primer, wherein the first primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of the first adapter oligonucleotides, and further wherein the second primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of the second adapter oligonucleotides. One or more of the primers used in the amplification step can comprise SEQ ID NO: 1. One or more of the primers used in the amplification step can comprise SEQ ID NO: 2.

[0012] In some embodiments, each second adapter oligonucleotide comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in the plurality of barcode sequences at at least three nucleotide positions. Pairs of first and second adapter oligonucleotides can comprise the same or different barcode sequences.

[0013] In some embodiments, the method further comprises sequencing one or more of the polynucleotides in a pool of target polynucleotides from independent samples. Sequencing can comprise extension of a sequencing primer comprising a sequence hybridizable to at least a portion of the complement of the first adapter oligonucleotide and/or the second adapter oligonucleotide. In some embodiments, the sequencing primer comprises SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, sequencing comprises a calibration step, wherein calibration is based on each of the nucleotides at one or more nucleotide positions in the barcode sequences.

[0014] In some embodiments, the method further comprises identifying the sample from which a target polynucleotide is derived based on a barcode sequence to which it is joined.

[0015] In another aspect, the invention provides compositions for use in the described methods, comprising any one or more of the elements described herein. In one aspect, the invention provides a composition for multiplex sequencing. In one embodiment, the composition comprises a plurality of target polynucleotides, each target polynucleotide comprising one or more barcode sequences selected from a plurality of barcode sequences, wherein said target polynucleotides are from two or more different samples, and further wherein the sample from which each of said polynucleotides is derived can be identified in a combined sequencing reaction with an accuracy of at least 95% based on a single barcode contained in the sequence of said target polynucleotide.

[0016] In another aspect, the invention provides a composition useful in the generation of adapter-tagged target polynucleotides, comprising any one or more of the elements described herein. In one embodiment, the composition comprises a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions. In some embodiments, the composition further comprises a plurality of second adapter oligonucleotides. In some embodiments, target polynucleotides are contained in a flow cell. First adapter oligonucleotides can be grouped in multiples of four such that all four bases are evenly represented at each position along each barcode. Where the second adapter oligonucleotide comprises a barcode, pairs of first and second adapter oligonucleotides can comprise the same or different barcode sequences. In some embodiments, the composition further comprises a first primer and a second primer, wherein said first primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of said first adapter oligonucleotides, and further wherein said second primers comprise a sequence that is hybridizable to at least a portion of the complement of one or more of said second adapter oligonucleotides. In some embodiments, the composition additionally comprises a sequencing primer comprising a sequence hybridizable to at least a portion of the complement of said first adapter oligonucleotide and/or said second adapter oligonucleotide.

[0017] In some embodiments, the composition comprises a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises a 5' end comprising sequence A and a 3'

end comprising sequence A', and further wherein A is hybridizable to A', one of A or A' comprises DNA, and the other of A or A' comprises RNA and 5 or more terminal DNA nucleotides. In some embodiments, the composition further comprises a plurality of second adapter oligonucleotides, wherein each of said second adapter oligonucleotides comprises a 5' end comprising sequence B and a 3' end comprising sequence B', and further wherein B is hybridizable to B', one of B or B' comprises DNA, and the other of B or B' comprises RNA and 5 or more terminal DNA nucleotides.

[0018] In another aspect, the invention provides kits containing any one or more of the elements disclosed in the described methods and compositions. In one aspect, the invention provides a kit useful in the generation of adapter-tagged target polynucleotides. In one embodiment, the kit comprises a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions, and instructions for using the same. In some embodiments, the kit further comprises a plurality of second adapter oligonucleotides. In some embodiments, the kit further comprises a first primer and a second primer, wherein said first primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of said first adapter oligonucleotides, and further wherein said second primers comprise a sequence that is hybridizable to at least a portion of the complement of one or more of said second adapter oligonucleotides. In some embodiments, the kit additionally comprises a sequencing primer comprising a sequence hybridizable to at least a portion of the complement of said first adapter oligonucleotide and/or said second adapter oligonucleotide. In some embodiments, the kit further comprises one or more of: (a) a DNA ligase, (b) a DNA-dependent DNA polymerase, (c) an RNA-dependent DNA polymerase, (d) random primers, (e) primers comprising at least 4 thymidines at the 3' end, (f) a DNA endonuclease, (g) a DNA-dependent DNA polymerase having 3' to 5' exonuclease activity, (h) a plurality of primers, each primer having one of a plurality of selected sequences, (i) a DNA kinase, (j) a DNA exonuclease, (k) magnetic beads, (l) an enzyme comprising RNase H activity, (m) an RNA ligase, and (n) one or more buffers suitable for one or more of the elements contained in said kit.

[0019] In some embodiments, the kit comprises a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises a 5' end comprising sequence A and a 3' end comprising sequence A', and further wherein A is hybridizable to A', one of A or A' comprises DNA, and the other of A or A' comprises RNA and 5 or more terminal DNA nucleotides. In some embodiments, the kit further comprises a plurality of second adapter oligonucleotides, wherein each of said second adapter oligonucleotides comprises a 5' end comprising sequence B and a 3' end comprising sequence B', and further wherein B is hybridizable to B', one of B or B' comprises DNA, and the other of B or B' comprises RNA and 5 or more terminal DNA nucleotides.

[0020] In another aspect, the invention provides a method of producing adapter-tagged polynucleotides. In one embodiment, the method comprises: (a) providing a plurality of first adapter oligonucleotides,

wherein each of said first adapter oligonucleotides comprises a 5' end comprising sequence A and a 3' end comprising sequence A', and further wherein A is hybridizable to A', one of A or A' comprises DNA, and the other of A or A' comprises RNA and 5 or more terminal DNA nucleotides; and, (b) joining at least one of said first adapter oligonucleotides to at least one of said target polynucleotides. Each of said first adapter oligonucleotides may comprise a barcode sequence. In some embodiments, the method further comprises the step of cleaving RNA with an enzyme that cleaves RNA from an RNA-DNA heteroduplex. In some embodiments, the method further comprises the step of extending one or more 3' ends of said target polynucleotides, using said one or more joined adapter oligonucleotides as template. In some embodiments, the method comprises joining at least one of a plurality of second adapter oligonucleotides to said target polynucleotides of each of said samples from step (b), such that at least one of said target polynucleotides comprises said first adapter oligonucleotide at one end and said second adapter oligonucleotide at the other end. In some embodiments, each of said second adapter oligonucleotides comprises a 5' end comprising sequence B and a 3' end comprising sequence B', and further wherein B is hybridizable to B', one of B or B' comprises DNA, and the other of B or B' comprises RNA and 5 or more terminal DNA nucleotides. In some embodiments, each of said second adapter oligonucleotides comprises a barcode sequence.

INCORPORATION BY REFERENCE

[0021] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0023] Figure 1 shows a schematic drawing of one embodiment of the methods of the invention.

[0024] Figure 2A shows an example result of amplification products obtained for target polynucleotides joined to adapter oligonucleotides, also referred to as "adapters," according to methods of the invention.

[0025] Figure 2B shows a side by side comparison of selected lanes from Fig. 2A, along with details about elements contained in the ligation reaction.

[0026] Figure 3 shows a schematic drawing of one embodiment of the methods of the invention, with hairpin adapters comprising RNA at the 5' end.

[0027] Figure 4 shows a schematic drawing of one embodiment of the methods of the invention, with hairpin adapters comprising RNA at the 3' end.

[0028] Figure 5 shows a schematic drawing of one embodiment of the methods of the invention, with hairpin adapters comprising RNA at the 3' end that are joined to a target polynucleotide, and further addition on non-hairpin adapters to ends of the target polynucleotide not joined to the hairpin adapter.

[0029] Figure 6 shows a schematic drawing of one embodiment of the methods of the invention.

[0030] Figure 7 shows various adapter designs, evaluated ligation efficiencies, and PCR amplified ligation products analyzed on an agarose gel.

[0031] Figure 8 shows an agarose gel containing target polynucleotides, adapter oligonucleotides, and ligation products.

[0032] Figure 9 shows an agarose gel containing PCR amplified ligation products.

[0033] Figure 10 shows a schematic drawing of one embodiment of the methods of the invention.

DEFINITIONS

[0034] The terms "polynucleotide", "nucleotide", "nucleotide sequence", "nucleic acid" and "oligonucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, intergenic DNA, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), small nucleolar RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Polynucleotide sequences, when provided, are listed in the 5' to 3' direction, unless stated otherwise.

[0035] As used herein, the term "target polynucleotide" refers to a nucleic acid molecule or polynucleotide in a starting population of nucleic acid molecules having a target sequence whose presence, amount, and/or nucleotide sequence, or changes in these, are desired to be determined. In general, a target polynucleotide is a double-stranded nucleic acid molecule, and may be derived from any source of or process for generating double-stranded nucleic acid molecules.

[0036] As used herein, the term "target sequence" refers generally to a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA, miRNA, and rRNA, or others. The target sequence may be a target sequence from a sample or a secondary target such as a product of an amplification reaction.

[0037] A "nucleotide probe," "probe," or "tag oligonucleotide" refers to a polynucleotide used for detecting or identifying its corresponding target polynucleotide in a hybridization reaction. Thus, a tag

oligonucleotide is hybridizable to one or more target polynucleotides. Tag oligonucleotides can be perfectly complementary to one or more target polynucleotides in a sample, or contain one or more nucleotides that are not complemented by a corresponding nucleotide in the one or more target polynucleotides in a sample.

[0038] “Hybridization” and “annealing” refer to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogsteen binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR, or the enzymatic cleavage of a polynucleotide by a ribozyme. A first sequence that can be stabilized via hydrogen bonding with the bases of the nucleotide residues of a second sequence is said to be “hybridizable” to said second sequence. In such a case, the second sequence can also be said to be hybridizable to the first sequence.

[0039] In general, a “complement” of a given sequence is a sequence that is fully complementary to and hybridizable to the given sequence. In general, a first sequence that is hybridizable to a second sequence or set of second sequences is specifically or selectively hybridizable to the second sequence or set of second sequences, such that hybridization to the second sequence or set of second sequences is preferred (e.g. thermodynamically more stable under a given set of conditions, such as stringent conditions commonly used in the art) to hybridization with non-target sequences during a hybridization reaction. Typically, hybridizable sequences share a degree of sequence complementarity over all or a portion of their respective lengths, such as between 25%-100% complementarity, including at least about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100% sequence complementarity.

[0040] The term “hybridized” as applied to a polynucleotide refers to a polynucleotide in a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogsteen binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self hybridizing strand, or any combination of these. The hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme. A sequence hybridized with a given sequence is referred to as the “complement” of the given sequence.

[0041] As used herein, “expression” refers to the process by which a polynucleotide is transcribed into mRNA and/or the process by which the transcribed mRNA (also referred to as “transcript”) is subsequently being translated into peptides, polypeptides, or proteins. The transcripts and the encoded polypeptides are collectively referred to as “gene product.” If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

DETAILED DESCRIPTION OF THE INVENTION

[0042] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See Sambrook, Fritsch and Maniatis, *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd edition (1989); *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY* (F. M. Ausubel, et al. eds., (1987)); the series *METHODS IN ENZYMOLOGY* (Academic Press, Inc.); *PCR 2: A PRACTICAL APPROACH* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) *ANTIBODIES, A LABORATORY MANUAL*, and *ANIMAL CELL CULTURE* (R.I. Freshney, ed. (1987)).

[0043] In one aspect, the present invention provides a method for multiplex sequencing. In one embodiment, the method comprises sequencing a plurality of target polynucleotides in a single reaction chamber, wherein said target polynucleotides are from two or more different samples; and identifying the sample from which each of said sequenced target polynucleotides is derived with an accuracy of at least 95% based on a single barcode contained in the sequence of said target polynucleotide. Reaction chambers can be any suitable chamber known in the art for containing a sequencing reaction, non-limiting examples of which include tubes of various dimensions, wells of multi-well plates, and channels of flow cells. In some embodiments, the target polynucleotides comprise one or more sequences with which the sequencing reaction is calibrated. In some embodiments, the one or more sequences with which the sequencing reaction is calibrated are joined to the target polynucleotides prior to sequencing.

[0044] In another aspect, the invention provides a method of producing adapter-tagged target polynucleotides from a plurality of independent samples. In one embodiment, the method comprises: (a) providing a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions; and (b) joining at least one of said first adapter oligonucleotides to said target polynucleotides of each of said samples, such that no barcode sequence is joined to said target polynucleotides of more than one of said samples. In some embodiments, the method further comprises (c) joining at least one of a plurality of second adapter oligonucleotides to said target polynucleotides of each of said samples from step (b), such that at least some of said target polynucleotides comprise said first adapter oligonucleotide at one end and said second adapter oligonucleotide at the other end. First and second adapter oligonucleotides can be the same or different, with different adapter oligonucleotides having different sequences and/or sequences of different lengths. A first adapter oligonucleotide can comprise one or more sequence regions that have the same sequence as one or more sequence regions of a second adapter oligonucleotide, and one or more sequence regions that have sequences that are different from one or more sequence regions of a second adapter oligonucleotide.

[0045] An adapter oligonucleotide includes any oligonucleotide having a sequence, at least a portion of which is known, that can be joined to a target polynucleotide. Adapter oligonucleotides can comprise

DNA, RNA, nucleotide analogues, non-canonical nucleotides, labeled nucleotides, modified nucleotides, or combinations thereof. Adapter oligonucleotides can be single-stranded, double-stranded, or partial duplex. In general, a partial-duplex adapter comprises one or more single-stranded regions and one or more double-stranded regions. Double-stranded adapters can comprise two separate oligonucleotides hybridized to one another (also referred to as an “oligonucleotide duplex”), and hybridization may leave one or more blunt ends, one or more 3' overhangs, one or more 5' overhangs, one or more bulges resulting from mismatched and/or unpaired nucleotides, or any combination of these. In some embodiments, a single-stranded adapter comprises two or more sequences that are able to hybridize with one another. When two such hybridizable sequences are contained in a single-stranded adapter, hybridization yields a hairpin structure (hairpin adapter). When two hybridized regions of an adapter are separated from one another by a non-hybridized region, a “bubble” structure results. Adapters comprising a bubble structure can consist of a single adapter oligonucleotide comprising internal hybridizations, or may comprise two or more adapter oligonucleotides hybridized to one another. Internal sequence hybridization, such as between two hybridizable sequences in an adapter, can produce a double-stranded structure in a single-stranded adapter oligonucleotide. Adapters of different kinds can be used in combination, such as a hairpin adapter and a double-stranded adapter, or adapters of different sequences. Hybridizable sequences in a hairpin adapter may or may not include one or both ends of the oligonucleotide. When neither of the ends are included in the hybridizable sequences, both ends are “free” or “overhanging.” When only one end is hybridizable to another sequence in the adapter, the other end forms an overhang, such as a 3' overhang or a 5' overhang. When both the 5'-terminal nucleotide and the 3'-terminal nucleotide are included in the hybridizable sequences, such that the 5'-terminal nucleotide and the 3'-terminal nucleotide are complementary and hybridize with one another, the end is referred to as “blunt.” Different adapters can be joined to target polynucleotides in sequential reactions or simultaneously. For example, the first and second adapters can be added to the same reaction. Adapters can be manipulated prior to combining with target polynucleotides. For example, terminal phosphates can be added or removed.

[0046] In some embodiments, one of the hybridizable sequences in a single-stranded hairpin adapter comprises RNA. For example, an adapter can comprise a 5' end comprising sequence A and a 3' end comprising sequence A', where A is hybridizable to A', one of A or A' comprises DNA, and the other of A or A' comprises RNA. Similarly, an adapter can comprise a 5' end comprising sequence B and a 3' end comprising sequence B', where B is hybridizable to B', one of B or B' comprises DNA, and the other of B or B' comprises RNA. In some embodiments, one of A or A' consists entirely of DNA, and/or one of A or A' consists entirely of RNA. In some embodiment, one of B or B' consists entirely of DNA, and/or one of B or B' consists entirely of RNA. Sequence A can be the same as or different from sequence B and/or B'. Sequence A' can be the same as or different from sequence B and/or B'. In some embodiments, the end of a hairpin comprising RNA (e.g. A, A', B, or B') further comprises one or more terminal DNA residues (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more terminal DNA

residues), such that the sequence comprising RNA is flanked by DNA residues at both ends (i.e. both the 5' end and the 3' end of the sequence comprising RNA). Hybridization of a sequence comprising RNA to a sequence comprising DNA creates an RNA-DNA heteroduplex. In some embodiments, RNA is cleaved by an enzyme that cleaves RNA from an RNA-DNA heteroduplex, such as enzymes comprising ribonuclease activity. Preferably, the enzyme comprising ribonuclease activity cleaves ribonucleotides in an RNA/DNA heteroduplex regardless of the identity and type of nucleotides adjacent to the ribonucleotide to be cleaved. It is preferred that the ribonuclease cleaves independent of sequence identity. Examples of suitable enzymes comprising ribonuclease activity for the methods and compositions of the invention are well known in the art, including ribonuclease H (RNase H) and enzymes comprising RNase H activity, e.g., Hybridase. In some embodiments, cleavage of RNA from an RNA-DNA heteroduplex removes all double-stranded character from a single-stranded hairpin adapter oligonucleotide, such that extension by a polymerase that uses the adapter as template requires no strand displacement step or strand displacement activity. In some embodiments, both ends of a hairpin adapter comprising one end comprising RNA are joined to a target polynucleotide, such that cleavage of the RNA from the RNA-DNA heteroduplex produces a 5' overhang or a 3' overhang. In some embodiments, an end comprising a 5' overhang produced by cleavage of RNA from an RNA-DNA heteroduplex is filled in by the extension of the produced 3' end using the 5' overhang as template.

[0047] In some embodiments, where hairpin adapters comprising 3' ends comprising RNA are joined to both 3' ends of a double-stranded target polynucleotide, cleavage of RNA from the RNA-DNA heteroduplex is followed by hybridization of oligonucleotides to the adapter sequences joined in the first step, and ligation of the hybridized oligonucleotides to the 5' ends of the double-stranded target polynucleotide to produce a target polynucleotide comprising non-complementary, single-stranded overhangs of both strands at both ends. Amplification of a double-stranded target polynucleotides comprising non-complementary, single-stranded overhangs on both strands at both ends can comprise the use of a first and second primer, wherein the first primer is hybridizable to one of the overhangs and the second primer is hybridizable to the complement of the overhang at the other end of the strand to which the first primer is hybridizable. Sequencing of double-stranded target polynucleotides comprising non-complementary, single-stranded overhangs on both strands at both ends can comprise the use of one or more sequencing primers hybridizable to one or more of the overhangs, or complements thereof. An illustrative example of the production of a double-stranded target polynucleotide comprising non-complementary, single-stranded overhangs on both strands at both ends is shown in Figure 5.

[0048] Adapters can contain one or more of a variety of sequence elements, including but not limited to, one or more amplification primer annealing sequences or complements thereof, one or more sequencing primer annealing sequences or complements thereof, one or more barcode sequences, one or more common sequences shared among multiple different adapters or subsets of different adapters, one or more restriction enzyme recognition sites, one or more overhangs complementary to one or more target polynucleotide overhangs, one or more probe binding sites (e.g. for attachment to a sequencing platform,

such as a flow cell for massive parallel sequencing, such as developed by Illumina, Inc.), one or more random or near-random sequences (e.g. one or more nucleotides selected at random from a set of two or more different nucleotides at one or more positions, with each of the different nucleotides selected at one or more positions represented in a pool of adapters comprising the random sequence), and combinations thereof. Two or more sequence elements can be non-adjacent to one another (e.g. separated by one or more nucleotides), adjacent to one another, partially overlapping, or completely overlapping. For example, an amplification primer annealing sequence can also serve as a sequencing primer annealing sequence. Sequence elements can be located at or near the 3' end, at or near the 5' end, or in the interior of the adapter oligonucleotide. When an adapter oligonucleotide is capable of forming secondary structure, such as a hairpin, sequence elements can be located partially or completely outside the secondary structure, partially or completely inside the secondary structure, or in between sequences participating in the secondary structure. For example, when an adapter oligonucleotide comprises a hairpin structure, sequence elements can be located partially or completely inside or outside the hybridizable sequences (the "stem"), including in the sequence between the hybridizable sequences (the "loop"). In some embodiments, the first adapter oligonucleotides in a plurality of first adapter oligonucleotides having different barcode sequences comprise a sequence element common among all first adapter oligonucleotides in the plurality. In some embodiments, all second adapter oligonucleotides comprise a sequence element common among all second adapter oligonucleotides that is different from the common sequence element shared by the first adapter oligonucleotides. A difference in sequence elements can be any such that at least a portion of different adapters do not completely align, for example, due to changes in sequence length, deletion or insertion of one or more nucleotides, or a change in the nucleotide composition at one or more nucleotide positions (such as a base change or base modification). In some embodiments, an adapter oligonucleotide comprises a 5' overhang, a 3' overhang, or both that is complementary to one or more target polynucleotides. Complementary overhangs can be one or more nucleotides in length, including but not limited to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more nucleotides in length. Complementary overhangs may comprise a fixed sequence. Complementary overhangs may comprise a random sequence of one or more nucleotides, such that one or more nucleotides are selected at random from a set of two or more different nucleotides at one or more positions, with each of the different nucleotides selected at one or more positions represented in a pool of adapters with complementary overhangs comprising the random sequence. In some embodiments, an adapter overhang is complementary to a target polynucleotide overhang produced by restriction endonuclease digestion. In some embodiments, an adapter overhang consists of an adenine or a thymine.

[0049] In some embodiments, one or more of the adapter oligonucleotides comprises SEQ ID NO: 1. In some embodiments, one or more of the adapter oligonucleotides comprises SEQ ID NO: 2. In some embodiments, the sequence element common among all first adapter oligonucleotides comprises SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the sequence element common among all second adapter oligonucleotides comprises SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, one of SEQ

ID NO: 1 or SEQ ID NO: 2 is common among all first adapter oligonucleotides and the other of SEQ ID NO: 1 or SEQ ID NO: 2 is common among all second adapter oligonucleotides. In some embodiments, one or more of the adapter oligonucleotides comprises SEQ ID NO: 3. In some embodiments, one or more of the adapter oligonucleotides comprises SEQ ID NO: 4. In some embodiments the 3'-most nucleotide of SEQ ID NO: 3 and/or SEQ ID NO: 4 is followed by one or more nucleotides of a barcode sequence.

[0050] In some embodiments, an adapter comprising an oligonucleotide duplex comprises an oligonucleotide comprising SEQ ID NO: 86 and/or an oligonucleotide comprising SEQ ID NO: 87. In some embodiments, an adapter comprising an oligonucleotide duplex comprises an oligonucleotide comprising SEQ ID NO: 88, and/or an oligonucleotide comprising SEQ ID NO: 89.

[0051] Adapter oligonucleotides can have any suitable length, at least sufficient to accommodate the one or more sequence elements of which they are comprised. In some embodiments, adapters are about, less than about, or more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, 100, 200, or more nucleotides in length. In some embodiments, the stem of a hairpin adapter is about, less than about, or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, or more nucleotides in length. Stems may be designed using a variety of different sequences that result in hybridization between the complementary regions on a hairpin adapter, resulting in a local region of double-stranded DNA. For example, stem sequences may be utilized that are from 15 to 18 nucleotides in length with equal representation of G:C and A:T base pairs. Such stem sequences are predicted to form stable dsDNA structures below their predicted melting temperatures of ~45°C. Sequences participating in the stem of the hairpin can be perfectly complementary, such that each base of one region in the stem hybridizes via hydrogen bonding with each base in the other region in the stem according to Watson-Crick base-pairing rules. Alternatively, sequences in the stem may deviate from perfect complementarity. For example, there can be mismatches and or bulges within the stem structure created by opposing bases that do not follow Watson-Crick base pairing rules, and/or one or more nucleotides in one region of the stem that do not have the one or more corresponding base positions in the other region participating in the stem. Mismatched sequences may be cleaved using enzymes that recognize mismatches. The stem of a hairpin can comprise DNA, RNA, or both DNA and RNA. In some embodiments, the stem and/or loop of a hairpin, or one or both of the hybridizable sequences forming the stem of a hairpin, comprise nucleotides, bonds, or sequences that are substrates for cleavage, such as by an enzyme, including but not limited to endonucleases and glycosylases. The composition of a stem may be such that only one of the hybridizable sequences forming the stem is cleaved. For example, one of the sequences forming the stem may comprise RNA while the other sequence forming the stem consists of DNA, such that cleavage by an enzyme that cleaves RNA in an RNA-DNA duplex, such as RNase H, cleaves only the sequence comprising RNA. The stem and/or loop of a hairpin can comprise non-canonical nucleotides (e.g. uracil), and/or methylated nucleotides. In some embodiments, one strand of a hairpin adapter stem comprises

SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the loop sequence of a hairpin adapter is about, less than about, or more than about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or more nucleotides in length.

[0052] As used herein, the term “barcode” refers to a known nucleic acid sequence that allows some feature of a polynucleotide with which the barcode is associated to be identified. In some embodiments, the feature of the polynucleotide to be identified is the sample from which the polynucleotide is derived. In some embodiments, barcodes are at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more nucleotides in length. In some embodiments, barcodes are shorter than 10, 9, 8, 7, 6, 5, or 4 nucleotides in length. In some embodiments, barcodes associated with some polynucleotides are of different length than barcodes associated with other polynucleotides. In general, barcodes are of sufficient length and comprise sequences that are sufficiently different to allow the identification of samples based on barcodes with which they are associated. In some embodiments, a barcode, and the sample source with which it is associated, can be identified accurately after the mutation, insertion, or deletion of one or more nucleotides in the barcode sequence, such as the mutation, insertion, or deletion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides. In some embodiments, each barcode in a plurality of barcodes differ from every other barcode in the plurality at at least three nucleotide positions, such as at least 3, 4, 5, 6, 7, 8, 9, 10, or more positions. In some embodiments, both the first adapter and the second adapter comprise at least one of a plurality of barcode sequences. In some embodiments, barcodes for second adapter oligonucleotides are selected independently from barcodes for first adapter oligonucleotides. In some embodiments, first adapter oligonucleotides and second adapter oligonucleotides having barcodes are paired, such that adapters of the pair comprise the same or different one or more barcodes. In some embodiments, the methods of the invention further comprise identifying the sample from which a target polynucleotide is derived based on a barcode sequence to which the target polynucleotide is joined. In general, a barcode comprises a nucleic acid sequence that when joined to a target polynucleotide serves as an identifier of the sample from which the target polynucleotide was derived.

[0053] In some embodiments, the plurality of barcode sequences from which barcode sequences are selected includes sequences selected from the group consisting of: AAA, TTT, CCC, GGG. In some embodiments, the plurality of barcode sequences from which barcode sequences are selected includes sequences selected from the group consisting of: AAAA, CTGC, GCTG, TGCT, ACCC, CGTA, GAGT, TTAG, AGGG, CCAT, GTCA, TATC, ATTT, CACG, GGAC, and TCGA. In some embodiments, the plurality of barcode sequences from which barcode sequences are selected includes sequences selected from the group consisting of: AAAAA, AACCC, AAGGG, AATTT, ACACG, ACCAT, ACGTA, ACTGC, AGAGT, AGCTG, AGGAC, AGTCA, ATATC, ATCGA, ATGCT, ATTAG, CAACT, CACAG, CAGTC, CATGA, CCAAC, CCCC, CCGGT, CCTTG, CGATA, CGCGC, CGGCG, CGTAT, CTAGG, CTCTT, CTGAA, CTTCC, GAAGC, GACTA, GAGAT, GATCG, GCATT, GCCGG, GCGCC, GCTAA, GGAAG, GGCCT, GGGGA, GGTTT, GTACA, GTCAC, GTGTG, GTTTT, TAATG, TACGT, TAGCA, TATAC, TCAGA, TCCTC, TCGAG, TCTCT, TGACC, TGCAA, TGGTT, TGTGG, TTAAT, TTCCG, TTGGC, and TTTTA.

[0054] The terms "joining" and "ligation" as used herein, with respect to two polynucleotides, such as an adapter oligonucleotide and a target polynucleotide, refers to the covalent attachment of two separate polynucleotides to produce a single larger polynucleotide with a contiguous backbone. Methods for joining two polynucleotides are known in the art, and include without limitation, enzymatic and non-enzymatic (e.g. chemical) methods. Examples of ligation reactions that are non-enzymatic include the non-enzymatic ligation techniques described in U.S. Pat. Nos. 5,780,613 and 5,476,930, which are herein incorporated by reference. In some embodiments, an adapter oligonucleotide is joined to a target polynucleotide by a ligase, for example a DNA ligase or RNA ligase. Multiple ligases, each having characterized reaction conditions, are known in the art, and include, without limitation NAD⁺-dependent ligases including tRNA ligase, Taq DNA ligase, *Thermus filiformis* DNA ligase, *Escherichia coli* DNA ligase, Tth DNA ligase, *Thermus scotoductus* DNA ligase (I and II), thermostable ligase, Ampligase thermostable DNA ligase, VanC-type ligase, 9° N DNA Ligase, Tsp DNA ligase, and novel ligases discovered by bioprospecting; ATP-dependent ligases including T4 RNA ligase, T4 DNA ligase, T3 DNA ligase, T7 DNA ligase, Pfu DNA ligase, DNA ligase I, DNA ligase III, DNA ligase IV, and novel ligases discovered by bioprospecting; and wild-type, mutant isoforms, and genetically engineered variants thereof. Ligation can be between polynucleotides having hybridizable sequences, such as complementary overhangs. Ligation can also be between two blunt ends. Generally, a 5' phosphate is utilized in a ligation reaction. The 5' phosphate can be provided by the target polynucleotide, the adapter oligonucleotide, or both. 5' phosphates can be added to or removed from polynucleotides to be joined, as needed. Methods for the addition or removal of 5' phosphates are known in the art, and include without limitation enzymatic and chemical processes. Enzymes useful in the addition and/or removal of 5' phosphates include kinases, phosphatases, and polymerases. In some embodiments, both of the two ends joined in a ligation reaction (e.g. an adapter end and a target polynucleotide end) provide a 5' phosphate, such that two covalent linkages are made in joining the two ends. In some embodiments, only one of the two ends joined in a ligation reaction (e.g. only one of an adapter end and a target polynucleotide end) provides a 5' phosphate, such that only one covalent linkage is made in joining the two ends. In some embodiments, only one strand at one or both ends of a target polynucleotide is joined to an adapter oligonucleotide. In some embodiments, both strands at one or both ends of a target polynucleotide are joined to an adapter oligonucleotide. In some embodiments, 3' phosphates are removed prior to ligation. In some embodiments, an adapter oligonucleotide is added to both ends of a target polynucleotide, wherein one or both strands at each end are joined to one or more adapter oligonucleotides. When both strands at both ends are joined to an adapter oligonucleotide, joining can be followed by a cleavage reaction that leaves a 5' overhang that can serve as a template for the extension of the corresponding 3' end, which 3' end may or may not include one or more nucleotides derived from the adapter oligonucleotide. In some embodiments, a target polynucleotide is joined to a first adapter oligonucleotide on one end and a second adapter oligonucleotide on the other end. In some embodiments, the target polynucleotide and the adapter to which it is joined comprise blunt ends. In some embodiments, separate

ligation reactions are carried out for each sample, using a different first adapter oligonucleotide comprising at least one barcode sequence for each sample, such that no barcode sequence is joined to the target polynucleotides of more than one sample. A target polynucleotide that has an adapter oligonucleotide joined to it is considered “tagged” by the joined adapter.

[0055] In some embodiments, joining of an adapter to a target polynucleotide produces a joined product polynucleotide having a 3' overhang comprising a nucleotide sequence derived from the adapter. In some embodiments, a primer oligonucleotide comprising a sequence complementary to all or a portion of the 3' overhang is hybridized to the overhang and extended using a DNA polymerase to produce a primer extension product hybridized to one strand of the joined product polynucleotide. The DNA polymerase may comprise strand displacement activity, such that one strand of the joined product polynucleotide is displaced during primer extension.

[0056] In some embodiments, after joining at least one adapter oligonucleotide to a target polynucleotide, the 3' end of one or more target polynucleotides is extended using the one or more joined adapter oligonucleotides as template. For example, an adapter comprising two hybridized oligonucleotides that is joined to only the 5' end of a target polynucleotide allows for the extension of the unjoined 3' end of the target using the joined strand of the adapter as template, concurrently with or following displacement of the unjoined strand. If both strands of an adapter comprising two hybridized oligonucleotides are joined to a target polynucleotide such that the joined product has a 5' overhang, the complementary 3' end can be extended using the 5' overhang as template. As a further example, a hairpin adapter oligonucleotide can be joined to the 5' end of a target polynucleotide. While double-stranded in secondary structure, such a hairpin adapter remains single-stranded, and is thus a 5' overhang appended to the target polynucleotide (e.g. when the 5' end of the hairpin adapter is not joined to the target polynucleotide). Removal of the secondary structure, either prior to (e.g. thermal denaturing, or degradation) or concurrently with (e.g. strand displacement) the activity of a polymerase, provides a template for the extension of the 3' end of the complementary strand of the target polynucleotide. In some embodiments, the 3' end of the target polynucleotide that is extended comprises one or more nucleotides from an adapter oligonucleotide. For target polynucleotides to which adapters are joined on both ends, extension can be carried out for both 3' ends of a double-stranded target polynucleotide having 5' overhangs. This 3' end extension, or “fill-in” reaction, generates a complementary sequence, or “complement,” to the adapter oligonucleotide template that is hybridized to the template, thus filling in the 5' overhang to produce a double-stranded sequence region. Where both ends of a double-stranded target polynucleotide have 5' overhangs that are filled in by extension of the complementary strands' 3' ends, the product is completely double-stranded. Extension can be carried out by any suitable polymerase known in the art, such as a DNA polymerase, many of which are commercially available. DNA polymerases can comprise DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity, or DNA-dependent and RNA-dependent DNA polymerase activity. DNA polymerases can be thermostable or non-thermostable. Examples of DNA polymerases include, but are

not limited to, Taq polymerase, Tth polymerase, Tli polymerase, Pfu polymerase, PfuTubo polymerase, Pyrobest polymerase, Pwo polymerase, KOD polymerase, Bst polymerase, Sac polymerase, Sso polymerase, Pcc polymerase, Pab polymerase, Mth polymerase, Pho polymerase, ES4 polymerase, VENT polymerase, DEEPVENT polymerase, EX-Taq polymerase, LA-Taq polymerase, Expand polymerases, Platinum Taq polymerases, Hi-Fi polymerase, Tbr polymerase, Tfl polymerase, Tru polymerase, Tac polymerase, The polymerase, Tma polymerase, Tih polymerase, Tfi polymerase, Klenow fragment, and variants, modified products and derivatives thereof. 3' end extension can be performed before or after pooling of target polynucleotides from independent samples.

[0057] In some embodiments, the fill-in reaction is followed by or performed as part of amplification of one or more target polynucleotides using a first primer and a second primer, wherein the first primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of the first adapter oligonucleotides, and further wherein the second primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of the second adapter oligonucleotides. Each of the first and second primers may be of any suitable length, such as about, less than about, or more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, 100, or more nucleotides, any portion or all of which may be complementary to the corresponding target sequence (e.g. about, less than about, or more than about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or more nucleotides). "Amplification" refers to any process by which the copy number of a target sequence is increased. Methods for primer-directed amplification of target polynucleotides are known in the art, and include without limitation, methods based on the polymerase chain reaction (PCR). Conditions favorable to the amplification of target sequences by PCR are known in the art, can be optimized at a variety of steps in the process, and depend on characteristics of elements in the reaction, such as target type, target concentration, sequence length to be amplified, sequence of the target and/or one or more primers, primer length, primer concentration, polymerase used, reaction volume, ratio of one or more elements to one or more other elements, and others, some or all of which can be altered. In general, PCR involves the steps of denaturation of the target to be amplified (if double stranded), hybridization of one or more primers to the target, and extension of the primers by a DNA polymerase, with the steps repeated (or "cycled") in order to amplify the target sequence. Steps in this process can be optimized for various outcomes, such as to enhance yield, decrease the formation of spurious products, and/or increase or decrease specificity of primer annealing. Methods of optimization are well known in the art and include adjustments to the type or amount of elements in the amplification reaction and/or to the conditions of a given step in the process, such as temperature at a particular step, duration of a particular step, and/or number of cycles. In some embodiments, an amplification reaction comprises at least 5, 10, 15, 20, 25, 30, 35, 50, or more cycles. In some embodiments, an amplification reaction comprises no more than 5, 10, 15, 20, 25, 35, 50, or more cycles. Cycles can contain any number of steps, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more steps. Steps can comprise any temperature or gradient of temperatures, suitable for achieving the purpose of the given step, including but not limited to, 3' end extension (e.g. adapter fill-in), primer annealing, primer

extension, and strand denaturation. Steps can be of any duration, including but not limited to about, less than about, or more than about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 180, 240, 300, 360, 420, 480, 540, 600, or more seconds, including indefinitely until manually interrupted. Cycles of any number comprising different steps can be combined in any order. In some embodiments, different cycles comprising different steps are combined such that the total number of cycles in the combination is about, less than about, or more than about 5, 10, 15, 20, 25, 30, 35, 50, or more cycles. In some embodiments, one or more of the primers comprises SEQ ID NO: 1. In some embodiments, one or more of the primers comprises SEQ ID NO: 2. In some embodiments, amplification is performed following the fill-in reaction. Amplification can be performed before or after pooling of target polynucleotides from independent samples.

[0058] In some embodiments, target polynucleotides from independent samples are pooled after the joining step. Pooling can be performed immediately after the joining step, or following one or more intervening steps between joining and pooling. Pools can comprise any fraction of the total target polynucleotides from a joining reaction, including the whole reaction volume. Samples can be pooled evenly or unevenly. Target polynucleotides can be further processed before or after pooling, for example to purify desired products or eliminate undesired products. Pools can comprise polynucleotides from any number of independent samples, such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 16, 20, 24, 28, 32, 36, 40, 50, 60, 70, 80, 90, 100, 128, 192, 384, 500, 1000 or more samples. In some embodiments, target polynucleotides are pooled based on the barcodes to which they are joined. In some embodiments, target polynucleotides from independent samples are pooled such that all four bases are evenly represented at one or more positions along the barcode, among barcodes included in the pool. In some embodiments, target polynucleotides from independent samples are pooled such that all four bases are evenly represented at every position along the barcode, among barcodes included in the pool. Where only one barcode is joined to polynucleotides of each sample, samples can be pooled in multiples of four in order to represent all four bases at one or more positions along the barcode evenly, for example 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 96, 128, 192, 256, 384, and so on. Where two barcodes are included in the joining reaction for each sample, such as two different first adapter oligonucleotides or one first adapter oligonucleotide and one second adapter oligonucleotide each having barcodes, samples can be pooled in multiples of two in order to evenly represent all four bases at one or more positions along the barcode, for example 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 48, 64, 96, 128, 256, 384, and so on. All combinations of the number of barcodes included in the joining reaction for target polynucleotides from each sample and the multiples in which samples are pooled in order to evenly represent all four nucleotides at one or more positions along the barcode are contemplated by the methods of the invention.

[0059] In some embodiments, pooling of target polynucleotides is followed by sequencing one or more polynucleotides in the pool. Sequencing processes are generally template dependent. Nucleic acid sequence analysis that employs template dependent synthesis identifies individual bases, or groups of

bases as they are added during a template mediated synthesis reaction, such as a primer extension reaction, where the identity of the base is complementary to the template sequence to which the primer sequence is hybridized during synthesis. Other such processes include ligation driven processes, where oligonucleotides or polynucleotides are complexed with an underlying template sequence, in order to identify the sequence of nucleotides in that sequence. Typically, such processes are enzymatically mediated using nucleic acid polymerases, such as DNA polymerases, RNA polymerases, reverse transcriptases, and the like, or other enzymes such as in the case of ligation driven processes, e.g., ligases.

[0060] Sequence analysis using template dependent synthesis can include a number of different processes. For example, in the ubiquitously practiced four-color Sanger sequencing methods, a population of template molecules is used to create a population of complementary fragment sequences. Primer extension is carried out in the presence of the four naturally occurring nucleotides, and with a sub-population of dye labeled terminator nucleotides, e.g., dideoxynucleotides, where each type of terminator (ddATP, ddGTP, ddTTP, ddCTP) includes a different detectable label. As a result, a nested set of fragments is created where the fragments terminate at each nucleotide in the sequence beyond the primer, and are labeled in a manner that permits identification of the terminating nucleotide. The nested fragment population is then subjected to size based separation, e.g., using capillary electrophoresis, and the labels associated with each different sized fragment is identified to identify the terminating nucleotide. As a result, the sequence of labels moving past a detector in the separation system provides a direct readout of the sequence information of the synthesized fragments, and by complementarity, the underlying template (See, e.g., U.S. Pat. No. 5,171,534, incorporated herein by reference in its entirety for all purposes).

[0061] Other examples of template dependent sequencing methods include sequence by synthesis processes, where individual nucleotides are identified iteratively, as they are added to the growing primer extension product.

[0062] Pyrosequencing is an example of a sequence by synthesis process that identifies the incorporation of a nucleotide by assaying the resulting synthesis mixture for the presence of by-products of the sequencing reaction, namely pyrophosphate. In particular, a primer/template/polymerase complex is contacted with a single type of nucleotide. If that nucleotide is incorporated, the polymerization reaction cleaves the nucleoside triphosphate between the α and β phosphates of the triphosphate chain, releasing pyrophosphate. The presence of released pyrophosphate is then identified using a chemiluminescent enzyme reporter system that converts the pyrophosphate, with AMP, into ATP, then measures ATP using a luciferase enzyme to produce measurable light signals. Where light is detected, the base is incorporated, where no light is detected, the base is not incorporated. Following appropriate washing steps, the various bases are cyclically contacted with the complex to sequentially identify subsequent bases in the template sequence. See, e.g., U.S. Pat. No. 6,210,891, incorporated herein by reference in its entirety for all purposes).

[0063] In related processes, the primer/template/polymerase complex is immobilized upon a substrate and the complex is contacted with labeled nucleotides. The immobilization of the complex may be through the primer sequence, the template sequence and/or the polymerase enzyme, and may be covalent or noncovalent. For example, immobilization of the complex can be via a linkage between the polymerase or the primer and the substrate surface. A variety of types of linkages are useful for this attachment, including, e.g., provision of biotinylated surface components, using e.g., biotin-PEG-silane linkage chemistries, followed by biotinylation of the molecule to be immobilized, and subsequent linkage through, e.g., a streptavidin bridge. Other synthetic coupling chemistries, as well as non-specific protein adsorption can also be employed for immobilization. In alternate configurations, the nucleotides are provided with and without removable terminator groups. Upon incorporation, the label is coupled with the complex and is thus detectable. In the case of terminator bearing nucleotides, all four different nucleotides, bearing individually identifiable labels, are contacted with the complex. Incorporation of the labeled nucleotide arrests extension, by virtue of the presence of the terminator, and adds the label to the complex. The label and terminator are then removed from the incorporated nucleotide, and following appropriate washing steps, the process is repeated. In the case of non-terminated nucleotides, a single type of labeled nucleotide is added to the complex to determine whether it will be incorporated, as with pyrosequencing. Following removal of the label group on the nucleotide and appropriate washing steps, the various different nucleotides are cycled through the reaction mixture in the same process. See, e.g., U.S. Pat. No. 6,833,246, incorporated herein by reference in its entirety for all purposes). For example, the Illumina Genome Analyzer System is based on technology described in WO 98/44151, hereby incorporated by reference, wherein DNA molecules are bound to a sequencing platform (flow cell) via an anchor probe binding site (otherwise referred to as a flow cell binding site) and amplified in situ on a glass slide. The DNA molecules are then annealed to a sequencing primer and sequenced in parallel base-by-base using a reversible terminator approach. Typically, the Illumina Genome Analyzer System utilizes flow-cells with 8 channels, generating sequencing reads of 18 to 36 bases in length, generating >1.3 Gbp of high quality data per run (see www.illumina.com).

[0064] In yet a further sequence by synthesis process, the incorporation of differently labeled nucleotides is observed in real time as template dependent synthesis is carried out. In particular, an individual immobilized primer/template/polymerase complex is observed as fluorescently labeled nucleotides are incorporated, permitting real time identification of each added base as it is added. In this process, label groups are attached to a portion of the nucleotide that is cleaved during incorporation. For example, by attaching the label group to a portion of the phosphate chain removed during incorporation, i.e., a β , γ , or other terminal phosphate group on a nucleoside polyphosphate, the label is not incorporated into the nascent strand, and instead, natural DNA is produced. Observation of individual molecules typically involves the optical confinement of the complex within a very small illumination volume. By optically confining the complex, one creates a monitored region in which randomly diffusing nucleotides are present for a very short period of time, while incorporated nucleotides are retained within the observation

volume for longer as they are being incorporated. This results in a characteristic signal associated with the incorporation event, which is also characterized by a signal profile that is characteristic of the base being added. In related aspects, interacting label components, such as fluorescent resonant energy transfer (FRET) dye pairs, are provided upon the polymerase or other portion of the complex and the incorporating nucleotide, such that the incorporation event puts the labeling components in interactive proximity, and a characteristic signal results, that is again, also characteristic of the base being incorporated (See, e.g., U.S. Pat. Nos. 6,056,661, 6,917,726, 7,033,764, 7,052,847, 7,056,676, 7,170,050, 7,361,466, 7,416,844 and Published U.S. Patent Application No. 2007-0134128, the full disclosures of which are hereby incorporated herein by reference in their entirety for all purposes).

[0065] In some embodiments, the nucleic acids in the sample can be sequenced by ligation. This method uses a DNA ligase enzyme to identify the target sequence, for example, as used in the polony method and in the SOLiD technology (Applied Biosystems, now Invitrogen). In general, a pool of all possible oligonucleotides of a fixed length is provided, labeled according to the sequenced position.

Oligonucleotides are annealed and ligated; the preferential ligation by DNA ligase for matching sequences results in a signal corresponding to the complementary sequence at that position.

[0066] In some embodiments, sequencing comprises extension of a sequencing primer comprising a sequence hybridizable to at least a portion of the complement of the first adapter oligonucleotide. In some embodiments, sequencing comprises extension of a sequencing primer comprising a sequence hybridizable to at least a portion of the complement of the second adapter oligonucleotide. A sequencing primer may be of any suitable length, such as about, less than about, or more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, 100, or more nucleotides, any portion or all of which may be complementary to the corresponding target sequence (e.g. about, less than about, or more than about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or more nucleotides). In some embodiments, the sequencing primer comprises SEQ ID NO: 1 or SEQ ID NO:2. In some embodiments, the sequencing primer comprises SEQ ID NO: 5. In some embodiments, the sequencing primer comprises SEQ ID NO: 6. In some embodiments, sequencing comprises a calibration step, wherein the calibration is based on each of the nucleotides at one or more nucleotide positions in the barcode sequences. Calibration can be useful in processing the sequencing data, for example, by facilitating or increasing the accuracy of identifying a base at a given position in the sequence.

[0067] In some embodiments, accurate identification of the sample from which a target polynucleotide is derived is based on at least a portion of the sequence obtained for the target polynucleotide and is at least 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.8%, 99.85%, 99.9%, 99.95%, 99.99%, or more accurate. In some embodiments, the sample source of a target polynucleotide is identified based on a single barcode contained in the sequence. In some embodiments, accuracy can be increased by identifying the source of a target polynucleotide using two or more barcodes contained in the sequence. Multiple barcodes can be joined to a target polynucleotide by the incorporation of multiple barcodes into a single adapter to which a target polynucleotide is joined, and/or by joining two or more adapters having one or more barcodes to a

target polynucleotide. In some embodiments, the identity of the sample source of a target polynucleotide comprising two or more barcode sequences may be accurately determined using only one of the barcode sequences that it comprises. In general, accurate identification of a sample from which a target polynucleotide is derived comprises correct identification of a sample source from among two or more samples in a pool, such as about, less than about, or more than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 16, 20, 24, 28, 32, 36, 40, 50, 60, 70, 80, 90, 100, 128, 192, 384, 500, 1000 or more samples in a pool.

[0068] The different samples from which the target polynucleotides are derived can comprise multiple samples from the same individual, samples from different individuals, or combinations thereof. In some embodiments, a sample comprises a plurality of polynucleotides from a single individual. In some embodiments, a sample comprises a plurality of polynucleotides from two or more individuals. An individual is any organism or portion thereof from which target polynucleotides can be derived, non-limiting examples of which include plants, animals, fungi, protists, monerans, viruses, mitochondria, and chloroplasts. Sample polynucleotides can be isolated from a subject, such as a cell sample, tissue sample, or organ sample derived therefrom, including, for example, cultured cell lines, biopsy, blood sample, or fluid sample containing a cell. The subject may be an animal, including but not limited to, an animal such as a cow, a pig, a mouse, a rat, a chicken, a cat, a dog, etc., and is usually a mammal, such as a human. Samples can also be artificially derived, such as by chemical synthesis. In some embodiments, the samples comprise DNA. In some embodiments, the samples comprise genomic DNA. In some embodiments, the samples comprise mitochondrial DNA, chloroplast DNA, plasmid DNA, bacterial artificial chromosomes, yeast artificial chromosomes, oligonucleotide tags, or combinations thereof. In some embodiments, the samples comprise DNA generated by primer extension reactions using any suitable combination of primers and a DNA polymerase, including but not limited to polymerase chain reaction (PCR), reverse transcription, and combinations thereof. Where the template for the primer extension reaction is RNA, the product of reverse transcription is referred to as complementary DNA (cDNA). Primers useful in primer extension reactions can comprise sequences specific to one or more targets, random sequences, partially random sequences, and combinations thereof. Reaction conditions suitable for primer extension reactions are known in the art. In general, sample polynucleotides comprise any polynucleotide present in a sample, which may or may not include target polynucleotides.

[0069] Methods for the extraction and purification of nucleic acids are well known in the art. For example, nucleic acids can be purified by organic extraction with phenol, phenol/chloroform/isoamyl alcohol, or similar formulations, including TRIzol and TriReagent. Other non-limiting examples of extraction techniques include: (1) organic extraction followed by ethanol precipitation, e.g., using a phenol/chloroform organic reagent (Ausubel et al., 1993), with or without the use of an automated nucleic acid extractor, e.g., the Model 341 DNA Extractor available from Applied Biosystems (Foster City, Calif.); (2) stationary phase adsorption methods (U.S. Pat. No. 5,234,809; Walsh et al., 1991); and (3) salt-induced nucleic acid precipitation methods (Miller et al., (1988), such precipitation methods being typically referred to as “salting-out” methods. Another example of nucleic acid isolation and/or

purification includes the use of magnetic particles to which nucleic acids can specifically or non-specifically bind, followed by isolation of the beads using a magnet, and washing and eluting the nucleic acids from the beads (see e.g. U.S. Pat. No. 5,705,628). In some embodiments, the above isolation methods may be preceded by an enzyme digestion step to help eliminate unwanted protein from the sample, e.g., digestion with proteinase K, or other like proteases. See, e.g., U.S. Pat. No. 7,001,724. If desired, RNase inhibitors may be added to the lysis buffer. For certain cell or sample types, it may be desirable to add a protein denaturation/digestion step to the protocol. Purification methods may be directed to isolate DNA, RNA, or both. When both DNA and RNA are isolated together during or subsequent to an extraction procedure, further steps may be employed to purify one or both separately from the other. Sub-fractions of extracted nucleic acids can also be generated, for example, purification by size, sequence, or other physical or chemical characteristic. In addition to an initial nucleic isolation step, purification of nucleic acids can be performed after any step in the methods of the invention, such as to remove excess or unwanted reagents, reactants, or products.

[0070] In some embodiments, sample polynucleotides are fragmented into a population of fragmented insert DNA molecules of one or more specific size range(s). In some embodiments, fragments are generated from at least about 1, 10, 100, 1000, 10000, 100000, 300000, 500000, or more genome-equivalents of starting DNA. Fragmentation may be accomplished by methods known in the art, including chemical, enzymatic, and mechanical fragmentation. In some embodiments, the fragments have an average length from about 10 to about 10,000 nucleotides. In some embodiments, the fragments have an average length from about 50 to about 2,000 nucleotides. In some embodiments, the fragments have an average length from about 100-2,500, 10-1,000, 10-800, 10-500, 50-500, 50-250, or 50-150 nucleotides. In some embodiments, the fragments have an average length less than 500 nucleotides, such as less than 400 nucleotides, less than 300 nucleotides, less than 200 nucleotides, or less than 150 nucleotides. In some embodiments, the fragmentation is accomplished mechanically comprising subsection sample polynucleotides to acoustic sonication. In some embodiments, the fragmentation comprises treating the sample polynucleotides with one or more enzymes under conditions suitable for the one or more enzymes to generate double-stranded nucleic acid breaks. Examples of enzymes useful in the generation of polynucleotide fragments include sequence specific and non-sequence specific nucleases. Non-limiting examples of nucleases include DNase I, Fragmentase, restriction endonucleases, variants thereof, and combinations thereof. For example, digestion with DNase I can induce random double-stranded breaks in DNA in the absence of Mg^{++} and in the presence of Mn^{++} . In some embodiments, fragmentation comprises treating the sample polynucleotides with one or more restriction endonucleases. Fragmentation can produce fragments having 5' overhangs, 3' overhangs, blunt ends, or a combination thereof. In some embodiments, such as when fragmentation comprises the use of one or more restriction endonucleases, cleavage of sample polynucleotides leaves overhangs having a predictable sequence. In some embodiments, the method includes the step of size selecting the fragments via standard methods such as column purification or isolation from an agarose gel.

[0071] In some embodiments, the 5' and/or 3' end nucleotide sequences of fragmented DNA are not modified prior to ligation with one or more adapter oligonucleotides. For example, fragmentation by a restriction endonuclease can be used to leave a predictable overhang, followed by ligation with one or more adapter oligonucleotides comprising an overhang complementary to the predictable overhang on a DNA fragment. In another example, cleavage by an enzyme that leaves a predictable blunt end can be followed by ligation of blunt-ended DNA fragments to adapter oligonucleotides comprising a blunt end. In some embodiments, the fragmented DNA molecules are blunt-end polished (or "end repaired") to produce DNA fragments having blunt ends, prior to being joined to adapters. The blunt-end polishing step may be accomplished by incubation with a suitable enzyme, such as a DNA polymerase that has both 3' to 5' exonuclease activity and 5' to 3' polymerase activity, for example T4 polymerase. In some embodiments, end repair is followed by an addition of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides, such as one or more adenine, one or more thymine, one or more guanine, or one or more cytosine, to produce an overhang. DNA fragments having an overhang can be joined to one or more adapter oligonucleotides having a complementary overhang, such as in a ligation reaction. For example, a single adenine can be added to the 3' ends of end repaired DNA fragments using a template independent polymerase, followed by ligation to one or more adapters each having a thymine at a 3' end. In some embodiments, adapter oligonucleotides can be joined to blunt end double-stranded DNA fragment molecules which have been modified by extension of the 3' end with one or more nucleotides followed by 5' phosphorylation. In some cases, extension of the 3' end may be performed with a polymerase such as for example Klenow polymerase or any of the suitable polymerases provided herein, or by use of a terminal deoxynucleotide transferase, in the presence of one or more dNTPs in a suitable buffer containing magnesium. In some embodiments, target polynucleotides having blunt ends are joined to one or more adapters comprising a blunt end. Phosphorylation of 5' ends of DNA fragment molecules may be performed for example with T4 polynucleotide kinase in a suitable buffer containing ATP and magnesium. The fragmented DNA molecules may optionally be treated to dephosphorylate 5' ends or 3' ends, for example, by using enzymes known in the art, such as phosphatases.

[0072] In some embodiments, each of the plurality of independent samples comprises at least about 1pg, 10pg, 100pg, 1ng, 10ng, 20ng, 30ng, 40ng, 50ng, 75ng, 100ng, 150ng, 200ng, 250ng, 300ng, 400ng, 500ng, 1µg, 1.5µg, 2µg, or more of nucleic acid material. In some embodiments, each of the plurality of independent samples comprises less than about 1pg, 10pg, 100pg, 1ng, 10ng, 20ng, 30ng, 40ng, 50ng, 75ng, 100ng, 150ng, 200ng, 250ng, 300ng, 400ng, 500ng, 1µg, 1.5µg, 2µg, or more of nucleic acid.

[0073] In another aspect, the invention provides compositions that can be used in the above described methods. Compositions of the invention can comprise any one or more of the elements described herein. In one embodiment, the composition comprises a plurality of target polynucleotides, each target polynucleotide comprising one or more barcode sequences selected from a plurality of barcode sequences, wherein said target polynucleotides are from two or more different samples, and further wherein the sample from which each of said polynucleotides is derived can be identified in a combined

sequencing reaction with an accuracy of at least 95% based on a single barcode contained in the sequence of said target polynucleotide. In some embodiments, the composition comprises a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions.

[0074] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. In some embodiments, a kit comprises a composition of the invention, in one or more containers. In some embodiments, the invention provides kits comprising adapters, primers, and/or other oligonucleotides described herein. In some embodiments, the kit further comprises one or more of: (a) a DNA ligase, (b) a DNA-dependent DNA polymerase, (c) an RNA-dependent DNA polymerase, (d) random primers, (e) primers comprising at least 4 thymidines at the 3' end, (f) a DNA endonuclease, (g) a DNA-dependent DNA polymerase having 3' to 5' exonuclease activity, (h) a plurality of primers, each primer having one of a plurality of selected sequences, (i) a DNA kinase, (j) a DNA exonuclease, (k) magnetic beads, (l) an enzyme comprising RNase H activity, (m) an RNA ligase, and (n) one or more buffers suitable for one or more of the elements contained in said kit. The adapters, primers, other oligonucleotides, and reagents can be, without limitation, any of those described above. Elements of the kit can further be provided, without limitation, in any of the amounts and/or combinations (such as in the same kit or same container) described above. The kits may further comprise additional agents, such as those described above, for use according to the methods of the invention. The kit elements can be provided in any suitable container, including but not limited to test tubes, vials, flasks, bottles, ampules, syringes, or the like. The agents can be provided in a form that may be directly used in the methods of the invention, or in a form that requires preparation prior to use, such as in the reconstitution of lyophilized agents. Agents may be provided in aliquots for single-use or as stocks from which multiple uses, such as in a number of reaction, may be obtained.

[0075] In one embodiment, the kit comprises a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions, and instructions for using the same. First adapters comprising different barcode sequences can be supplied individually or in combination with one or more additional first adapter having a different barcode sequence. In some embodiments, the kit further comprises a plurality of second adapter oligonucleotides. Second adapter oligonucleotides can be supplied separately from or in combination with one or more first adapters, and/or one or more different second adapters. Combinations of first and second adapters can be supplied in accordance with combinations described above.

EXAMPLES

[0076] The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. The present examples, along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

Example 1: Fragmentation and repair of sample nucleic acid

[0077] The sample comprising target polynucleotides ("sample") used in this example is human genomic DNA. In order to fragment the nucleic acid, 1 μ g to 5 μ g are diluted in 120 μ L of TE, and the dilution is subjected to mechanic fragmentation using a Covaris S-series sonication instrument (Covaris, Inc.) with the following setting: duty cycle = 10, intensity = 5, cycles/burst = 100, time = 10 minutes, and sample volume = 120 μ L. Fragmented nucleic acid is purified with SPRI beads (Beckman Coulter, Inc.) at a ratio of 1:1.8 (sample:beads). DNA is eluted from the beads with 40 μ L of TE, and quantified, such as by using a Nanodrop, Qubit, or similar DNA quantitation instrument, or spectrophotometrically. Fragmentation products having 5' overhangs, 3' overhangs, non-phosphorylated 3' ends, and/or phosphorylated 3' ends are then end-repaired using a blend of enzymes that specifically eliminate overhangs and restore end residues to the appropriate 5' phosphorylation and 3' hydroxyl configuration. For end-repair using the Quick Blunting kit (New England Biolabs, Inc.), 100-200ng of fragmented DNA is combined with 1.25 μ L 10X quick blunt buffer, 1.25 μ L 1mM dNTP mix, and water to a final volume of 12 μ L. This combination is mixed well, spun down in the tube, and 0.5 μ L of quick blunt enzyme (a combination of T4 DNA polymerase and T4 polynucleotide kinase) is added, followed by incubation at room temperature for 30 minutes, and inactivation at 70°C for 10 minutes. Nucleic acid prepared according to the methods of this example can be stored at -20°C or used immediately in subsequent ligation reaction to join target polynucleotide fragments to adapters. A diagram of the steps in the procedure, including fragmentation, end-repair, adapter ligation, adapter fill-in, amplification, and sequencing is shown in Figure 1.

Example 2: Effects of target polynucleotide to adapter ratios on library construction

[0078] The present example examines the effects of varying target polynucleotide to adapter ratios on the construction of a collection of adapter tagged target polynucleotides (or "library"). The sample comprising target polynucleotides ("sample") used in this example is prepared as described in Example 1. The first adapter in this example consists of SEQ ID NO: 7. The second adapter consists of SEQ ID NO: 8. One of the primers used in the amplification step of this example consists of SEQ ID NO: 9, and the other primer in the pair consists of SEQ ID NO: 10. Ligation reactions are prepared such that each

contains 10uL of 2X ligation buffer, 4uL of sample nucleic acid, 4uL of combined adapters, 1uL of water (5uL in reaction lacking sample or adapters), and 1uL ligase. In addition to buffer, water, and ligase, the tested reactions consist of: no sample (reactions 1 to 4), 20ng of sample (reaction 5 to 8), and 200ng of sample (reactions 9 to 12) in combination with (in reaction order) 1uM adapters, 0.2uM adapters, 0.04uM adapters, or 0.008uM adapters. Additional controls, in addition to buffer, water, and ligase, consist of the following, listed by reaction number: (13) 200ng of sample without adapters, (14) 200ng of sample and 1uM first adapter only, (15) 200ng of sample and 1uM second adapter only, (16) water only, (17) 1uM first adapter only, and (18) 1uM second adapter only. Ligation reactions are incubated at room temperature for 10 minutes. Ligation products are then subjected to an amplification step, with each amplification reaction containing 3uL water, 2uL 5X PCR buffer, 1uL 25mM MgCl₂, 1uL 10uM first primer, 1uL 10uM second primer, 0.5uL 10mM dNTPs, 0.5uM DMSO, 0.1uL Expand enzyme mix, 0.1uL Taq polymerase, and 1uL of one of the ligation reactions. Amplification reaction mixtures were then subjected to the following thermal cycling sequence: 1 cycle of 72°C for 2 minutes, 95°C for 2 minutes; 10 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute; 20 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 70°C seconds; 72°C for 7 minutes; hold at 10°C until proceeding. The first cycle in this process serves to extend the 3' ends of target polynucleotides using the adapters ligated to the 5' end as template (the "fill in" reaction), yielding a double-stranded DNA adapter tag. At the end of thermal cycling, 2uL of 6X loading dye is added to each reaction, and 5uL of the resulting mixture is loaded on a 2% agarose gel in TAE. Gels are imaged to illuminate the DNA products resulting from ligation and amplification.

[0079] A sample result is shown in Fig. 2A. The top half of Fig. 2A contains, in lanes from left to right, ladder, reactions 1 to 9, and ladder. The lower half of Fig. 2A contains, in lanes from left to right, ladder, reactions 10 to 18, and ladder. Lanes 1 to 4 and 13 to 18 show that both sample nucleic acid and both adapters are required for efficient amplification of target polynucleotides. Fig. 2B provides a side-by-side comparison of reactions 1 to 12, in order from left to right, in addition to lanes containing ladder. The results indicate that, under these conditions, amplified libraries can be obtained using a first and second hairpin adapter, that the higher sample amount decreases the formation of primer dimers, and amplification yield remains relatively constant with decreasing adapter input.

Example 3: Barcoded adapters and sample source identification

[0080] Nucleic acid is isolated from samples derived from 16 individuals using standard methods. Isolated polynucleotide samples are processed independently as in Example 1. Adapters are then ligated to target polynucleotides as in Example 2, with each sample being joined to a first adapter having a different barcode and a second adapter consisting of SEQ ID NO: 8. The first adapters are independently assigned to each of the samples, and have sequences provided by SEQ ID NOs: 11-26.

[0081] Target polynucleotides having 5' overhangs comprising the adapter sequences are then filled in by 3' end extension using the adapter sequences as template as in Example 2. Target polynucleotides are

then also PCR amplified as in Example 2, using a pair of primers, one comprising SEQ ID NO: 84 and the other comprising SEQ ID NO: 85. The amplification products are then pooled and submitted for sequencing according to Illumina's Solexa sequencing platform (see e.g. www.illumina.com). Pooled sequencing data is then parsed on the basis of the barcodes contained in the sequencing reads to create 16 bins of sequencing data. The bins are then assembled as though each had been run independently, providing sorted and aligned sequencing data for 16 independent samples from a single pooled sequencing reaction.

Example 4: Use of hairpin adapters comprising heteroduplexes

[0082] The sample comprising target polynucleotides ("sample") used in this example is prepared as described in Example 1. First and second hairpin adapter oligonucleotides having stems involving both ends, forming blunt-ended structures, are ligated to target polynucleotides as in Example 2. With only target polynucleotides having 5' phosphates, only the 3' ends of the adapters are joined to the targets. As illustrated in Fig. 3, the hybridizable region of the 5' ends of the adapters comprise RNA, while the sequence to which the 5' ends hybridize comprises DNA. After ligation, RNase H cleaves the RNA of the RNA-DNA heteroduplexes, removing the secondary structure from the ligated adapters. DNA polymerase then extends the 3' ends of the target polynucleotides using the remaining sequence of the ligated adapters as template, a step that does not require any strand displacement. This step is performed as in Example 2, and can also be followed by an amplification step using primers that hybridize to sequences derived from the adapters. The resulting adapter-tagged oligonucleotides are then sequenced using sequencing primers that hybridize to sequences derived from the adapters. In both Fig. 3 and Fig. 4, S1 (half of stem 1) is hybridizable to S1' (the other half of stem 1), S2 (half of stem 2) is hybridizable to S2' (the other half of stem 2), L1 is the loop sequence of the first adapter oligonucleotide, and L2 is the loop sequence of the second adapter oligonucleotide. Similarly, in Fig. 5, S1 is hybridizable to S1', and L1 is the loop sequence of the adapter oligonucleotide. For the purpose of these illustrations, the sequences S1, S1', S2, and S2' correspond to sequences A, A', B, and B', respectively, as discussed above.

Example 5: Evaluation ligation efficiency of various hairpin adapter designs

[0083] In this example, hairpin adapter oligonucleotides having different nucleotide compositions were evaluated for efficiency of ligation to target polynucleotides. Each ligation reaction included target polynucleotides and a pair of adapters, with each member of the pair having a different sequence but sharing a specified characteristic. As illustrated in Figure 7, the various designs are, from left to right, blunt dU adapters, thymine-overhang adapters (ligated to blunt-ended target polynucleotides), thymine-overhang adapters (ligated to end repaired target polynucleotides modified to have 3' adenine single-base overhangs), duplex hairpin adapters, and blunt all-DNA adapters. Blunt dU adapters included a dinucleotide of deoxy uridine nucleotides at the 5' extreme end of the adapter loop (e.g. SEQ ID NO: 27

and SEQ ID NO: 28). Treatment of ligated material with UDG + APE1 cleaves the U bases and opens the loop for a subsequent fill in reaction (the remaining stem dissociates at the 72°C temperature used in the fill-in reaction. Thymine-overhang adapters included an all-DNA sequence with a single thymine nucleotide 3' overhang (e.g. SEQ ID NO: 35 and SEQ ID NO 36). Duplex hairpin adapters included a first or second hairpin oligonucleotide having a stem and a 3' overhang (e.g. SEQ ID NO: 37 and SEQ ID NO: 38) hybridized to a short nucleotide (e.g. SEQ ID NO: 39), which hybridization included the 5' terminus of the short nucleotide and the 3' terminus of the hairpin oligonucleotide to form a stem effectively having a single-stranded break. Blunt all-DNA adapters consisted of DNA, with internal hybridization forming a blunt ended hairpin (e.g. SEQ ID NO: 40 and SEQ ID NO: 41). Example adapter sequences are provided by SEQ ID NOs: 27-43.

[0084] Human genomic DNA was fragmented as in Example 1. For end repair of fragmented genomic DNA, 52uL of 191ng/uL fragmented human genomic DNA was combined with 20uL 10X quick blunt buffer, 20uL 10X dNTPs, and 100uL water, which was mixed before further adding 8uL quick blunt enzyme mix. The end-repair reaction was incubated for 30 minutes at room temperature, and for 20 minutes at 75°C. For ligation to thymine-overhang adapters, 100uL of end-repaired DNA was modified to have single adenine nucleotide 3' overhangs ("tailed") by adding 2uL of 10mM dATP (final concentration of 0.2mM) and 8uL of Klenow (3'→5' exo minus), and incubating at 37°C for 30 minutes followed by 75°C for 20 minutes. Ligation reactions were prepared by combining 10uL of 2X ligation buffer, 4uL of end-repaired DNA or tailed DNA (approximately 200ng total), 0.2uL of each of a first and second adapter in a pair at concentrations of 10uM, and 5uL water, followed by mixing, adding 1uL of T4 DNA ligase, and incubating at room temperature for 10 minutes. For ligation reactions using blunt dU adapters, 1uL of a mixture of uracil DNA glycosylase (UDG) and apurinic endonuclease (APE) was added, followed by incubation at 37°C for 10 minutes. After ligation, and cleavage where indicated, duplicate reactions were prepared for fill-in of 5' overhangs by 3' end extension for each adapter type ligation reaction. One of each duplicate fill-in reaction was further amplified by PCR using a pair of amplification primers (SEQ ID NO: 42 and SEQ ID NO: 43), while the other of each duplicate was used in the determination of ligation efficiency. Each fill-in/amplification reaction contained 8uL of water, 2uL of 10X amplification buffer, 2uL of 25mM MgCl₂, 2uL of each amplification primer at 10uM concentrations, 2uL of one ligation reaction, 1uL of DMSO, 1uL of 10mM dNTPs, and 0.2uL of Taq polymerase enzyme. The fill-in/amplification reactions were incubated at 72°C for 2 minutes. Amplification included 20 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Aliquots of amplification reactions were run on an agarose gel, the results of which are illustrated in Figure 7.

[0085] Ligation efficiency was measured by quantitative PCR (qPCR). Ligation efficiency is defined as the percent of target molecules that are added to the library construction as input that end up in the final, amplified library. It is determined by using an existing library of known composition and concentration as a standard. Dilutions of this library are used to create a standard curve in a qPCR reaction. For

measurement of unknowns, a calculated portion of the target input is removed following end-repair, ligation, and fill in. The qPCR signal from this sample is plotted on the standard curve to establish the quantity of properly ligated molecules. The difference between the measured signal and the known input establishes the ligation efficiency. qPCR reaction mixes included 12.5uL of 2X SYBR mix (Clontech Laboratories, Inc.), 0.5uL of each amplification primer at 10uM concentrations, 5uL of template (1/10 dilution of fill-in reaction, 1/100 dilution of fill-in reaction, library standard, or water for no template control), and 6.5uL of water. Amplification of the qPCR reactions was conducted using standard methods, and ligation efficiency for each adapter design is provide below the illustration of the respective design in Figure 7. In summary, for blunt dU adapters, thymine-overhang adapters (ligated to blunt-ended target polynucleotides), thymine-overhang adapters (ligated to end repaired target polynucleotides modified to have 3' adenine single-base overhangs), duplex hairpin adapters, and blunt all-DNA adapters, efficiencies were about 0.48%, 0.0035%, 0.20%, 0.22%, and 0.22%, respectively. All adapter pairs produced comparable PCR amplification products.

[0086] Examination of ligation products by agarose gel analysis indicated the presence of little or no adapter dimers. That amplification products contained target insert fragments of about the expected size was also confirmed. Figure 8 shows a gel of samples of various reactions, with lane contents from left to right as follows: end repaired human genomic DNA, blunt all-DNA adapters, end repaired and A-tailed DNA, thymine overhang adapters, ladder, ligated end repaired DNA without adapters, end repaired DNA ligated with blunt all-DNA adapters, end repaired and A-tailed DNA ligated without adapters, end repaired and A-tailed DNA ligated with thymine overhang adapters, and ladder.

[0087] In some embodiments, a first duplex adapter in a pair of duplex adapters includes a first hairpin oligonucleotide having a stem and a 3' overhang comprising a barcode hybridized to a short partner oligonucleotide comprising a sequence complementary to all or a portion of the 3' overhang, including the barcode. The duplex adapter comprising two oligonucleotides may have a 5' or 3' overhang, or a blunt end when both oligonucleotides in the duplex are hybridized. The first duplex adapter may be paired with a second duplex adapter that is the same or different from the first duplex adapter, and the second duplex adapter may or may not contain a barcode. In general, the second duplex adapter may include a hairpin oligonucleotide having a stem and a 3' overhang hybridized to a short nucleotide, such that the hybridized oligonucleotides together form an adapter having a 5' or 3' overhang, or a blunt end. Examples of first duplex adapters comprising a hairpin oligonucleotide having a barcode and paired with a short partner oligonucleotide includes the following sequence pairs: SEQ ID NO: 44 with SEQ ID NO: 45, SEQ ID NO: 46 with SEQ ID NO: 47, SEQ ID NO: 48 with SEQ ID NO: 49, SEQ ID NO: 50 with SEQ ID NO: 51, SEQ ID NO: 52 with SEQ ID NO: 53, SEQ ID NO: 54 with SEQ ID NO: 55, SEQ ID NO: 56 with SEQ ID NO: 57, SEQ ID NO: 58 with SEQ ID NO: 59, SEQ ID NO: 60 with SEQ ID NO: 61, SEQ ID NO: 62 with SEQ ID NO: 63, SEQ ID NO: 64 with SEQ ID NO: 65, SEQ ID NO: 66 with SEQ ID NO: 67, SEQ ID NO: 68 with SEQ ID NO: 69, SEQ ID NO: 70 with SEQ ID NO: 71, SEQ ID NO: 72 with SEQ ID NO: 73, and SEQ ID NO: 74 with SEQ ID NO: 75. In these sequences, barcodes

are represented by the four bases at the 3' end of the hairpin oligonucleotide in each pair of oligonucleotides of the duplex adapter, and the complement of the barcode represented by the four bases at the 5' end of the short partner oligonucleotide in each pair of oligonucleotides of the duplex adapter. In general, each hairpin oligonucleotide of a pair is mixed with the corresponding short partner oligonucleotide in a ratio of 1:1.

Example 6: Evaluation ligation efficiency of hairpin adapters comprising RNA

[0088] In this example, hairpin adapter oligonucleotides having different nucleotide compositions were evaluated for efficiency of ligation to target polynucleotides, as in Example 5. Each ligation reaction included target polynucleotides and a pair of adapters, with each member of the pair having a different sequence but sharing a specified characteristic. The adapter pairs included blunt all-DNA adapters, and blunt RNA with DNA:DNA end adapters. Blunt all-DNA adapters consisted of DNA, with internal hybridization forming a blunt ended hairpin (SEQ ID NO: 76 and SEQ ID NO: 77). Blunt RNA with DNA:DNA end adapters included a stem having one strand with 10 RNA bases at the 5' end with five 5' terminal DNA bases, hybridized to a second strand of all DNA (SEQ ID NO: 80 and SEQ ID NO: 81). Amplification of ligation reactions using these adapters was performed using a pair of amplification primers (SEQ ID NO: 82 and SEQ ID NO: 83). Example adapter and amplification primer sequences are provided by SEQ ID NOs: 76-83.

[0089] Fragmented target polynucleotides were prepared as in Example 5. Fragmented DNA was end-repaired as in Example 1 by combining, for each reaction, 4.2uL of 47.5ng/uL fragmented genomic DNA, 1.25uL of 10X quick blunt buffer, 1.25uL of 1mM dNTP, 5.3uL water, mixing, and adding 0.5uL quick blunt enzyme. End-repair reactions were then incubated at room temperature (e.g. 20°C-27°C) for 30 minutes, and then incubated at 70°C for 10 minutes. Ligation reactions were prepared in duplicate, using a full 12.5uL end-repair reaction, combined with 12.5uL of 2X quick ligase buffer, 0.25uL of each adaptor in a pair at concentrations of 10uM, and 1.25uL of quick ligase. Ligation reactions were incubated at room temperature for 10 minutes prior to amplification. One ligation reaction in each duplicate was treated with RNase H in the amplification reaction mixture before beginning the amplification process. Both RNase H treated and untreated reactions were then subjected to 5' overhang fill-in and ligation product amplification. Samples not treated with RNase H included 59uL of water, 10uL of 10x PCR buffer, 3uL of 50mM MgCl₂, 5uL of each amplification primer at concentrations of 10uM, 5uL of DMSO, 2uL of 10mM dNTPs, 1uL of Taq polymerase, and 10uL of ligated template. Samples receiving RNase H treatment included 58uL of water, 10uL of 10x PCR buffer, 3uL of 50mM MgCl₂, 5uL of each amplification primer at concentrations of 10uM, 5uL of DMSO, 2uL of 10mM dNTPs, 1uL of Taq polymerase, 1uL of RNase H, and 10uL of ligated template. For samples receiving RNase H treatment, thermal cycling for amplification was preceded by incubation at 37°C for 10 minutes (non-amplified, RNase H treated samples used as a baseline for quantification included additional steps of 72°C for 2 minutes, and a 10°C hold step). Amplification reaction mixtures were then subjected to the

following thermal cycling sequence for fill-in and amplification: 1 cycle of 72°C for 2 minutes; 20 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 90 seconds; 1 cycle of 72°C for 7 minutes; and rest at 10°C. A 2% agarose gel containing 8uL samples of PCR amplification reactions is shown in Figure 9, with lanes from left to right corresponding to blunt RNA with DNA:DNA end adapter ligation products, blunt all-DNA adapter ligation products, and DNA ladder. A schematic diagram of the ligation between the 3' end of the adapter and 5' end of the target, RNase H treatment (where applicable), fill-in, and amplification reactions at one end of a target DNA is provided in Figure 10.

[0090] Ligation efficiency was measured for each pair of adapters with or without RNase H treatment as described in Example 5. Each qPCR reaction in this example comprised 5uL of 2X SYBR Green Mix, 0.4uL of each amplification primer, 2.2uL water, and 2uL of diluted ligation reaction, for a total of 10uL for each qPCR reaction. Ligation efficiencies for blunt all-DNA adapters with RNase H treatment, blunt all-DNA adapters without RNase H treatment, blunt RNA with DNA:DNA end adapters with RNase H treatment, and blunt RNA with DNA:DNA end adapters without RNase H treatment were 0.20%, 0.37%, 0.28%, and 0.13%, respectively. Successfully ligated and amplified fragments may be used as a next-generation sequence library.

[0091] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for multiplex sequencing comprising sequencing a plurality of target polynucleotides in a single reaction chamber, wherein said target polynucleotides are from two or more different samples; and identifying the sample from which each of said sequenced target polynucleotides is derived with an accuracy of at least 95% based on a single barcode contained in the sequence of said target polynucleotide.
2. The method of claim 1, wherein said target polynucleotides comprise one or more sequences with which the sequencing reaction is calibrated.
3. The method of claim 1, wherein each barcode differs from every other barcode at at least three nucleotide positions.
4. The method of claim 1, wherein said identification is accurate after the mutation or deletion of a nucleotide in said barcode.
5. A method of producing adapter-tagged target polynucleotides from a plurality of independent samples, the method comprising:
 - a) providing a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions; and
 - b) joining at least one of said first adapter oligonucleotides to said target polynucleotides of each of said samples, such that no barcode sequence is joined to said target polynucleotides of more than one of said samples.
6. The method of claim 5, further comprising (c) joining at least one of a plurality of second adapter oligonucleotides to said target polynucleotides of each of said samples from step (b), such that at least some of said target polynucleotides comprise said first adapter oligonucleotide at one end and said second adapter oligonucleotide at the other end.
7. The method of claim 6, further comprising pooling the target polynucleotides from step (c).
8. The method of claim 7, further comprising sequencing one or more of said polynucleotides in said pool.
9. The method of claim 8, further comprising identifying the sample from which a target polynucleotide is derived based on a barcode sequence to which it is joined.
10. The method of claims 5 or 6, wherein one or more of said adapter oligonucleotides comprises SEQ ID NO: 1.
11. The method of claims 5 or 6, wherein one or more of said adapter oligonucleotides comprises SEQ ID NO: 2.

12. The method of claims 5 or 6, wherein one or more of said adapter oligonucleotides comprise a hairpin structure.
13. The method of claims 5 or 6, wherein one or more of said adapter oligonucleotides comprise an oligonucleotide duplex.
14. The method of claims 1 or 5, wherein said barcode sequences are at least 3 nucleotides in length.
15. The method of claims 1 or 7, wherein said target polynucleotides are pooled based on said barcode sequences, such that all four bases are evenly represented at one or more positions along each barcode in said pool.
16. The method of claims 1 or 5, wherein said target polynucleotides comprise fragmented sample polynucleotides.
17. The method of claim 16, wherein said fragmentation comprises subjecting said sample polynucleotides to acoustic sonication.
18. The method of claim 16, wherein said fragmentation comprises treating said sample polynucleotides with one or more restriction endonucleases.
19. The method of claim 16, wherein said fragmentation comprises treating said sample polynucleotides with one or more enzymes under conditions suitable for said one or more enzymes to generate random double-stranded nucleic acid breaks.
20. The method of claim 19, wherein said one or more enzymes are selected from the group consisting of: DNase I, Fragmentase, and variants thereof.
21. The method of claim 16, wherein said fragments have an average length of 10 to 10000 nucleotides.
22. The method of claim 16, wherein said fragments have an average length of 100-2500 nucleotides.
23. The method of claim 16, wherein said fragments have an average length of 50-500 nucleotides.
24. The method of claims 12 or 13, further comprising performing the step of extending one or more 3' ends of said target polynucleotides, using said one or more joined adapter oligonucleotides as template.
25. The method of claim 24, further comprising amplifying said target polynucleotides after said extending step using a first primer and a second primer, wherein said first primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of said first adapter oligonucleotides, and further wherein said second primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of said second adapter oligonucleotides.
26. The method of claim 25, wherein one or more of said primers comprises SEQ ID NO: 1.
27. The method of claim 25, wherein one or more of said primers comprises SEQ ID NO: 2.

28. The method of claim 6, wherein each of said second adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions.

29. The method of claim 28, wherein pairs of said first and second adapter oligonucleotides comprise different barcode sequences.

30. The method of claim 28, wherein pairs of said first and second adapter oligonucleotides comprise the same barcode sequence.

31. The method of claims 1 or 5, wherein said target polynucleotides comprise genomic DNA.

32. The method of claims 1 or 5, wherein said target polynucleotides comprise mitochondrial DNA, chloroplast DNA, plasmid DNA, bacterial artificial chromosomes, yeast artificial chromosomes, or a combination thereof.

33. The methods of claims 1 or 5, wherein said target polynucleotides comprise cDNA.

34. The method of claims 1 or 5, wherein said samples comprise target polynucleotides produced by a primer extension reaction.

35. The method of claim 8, wherein said sequencing comprises extension of a sequencing primer comprising a sequence hybridizable to at least a portion of the complement of said first adapter oligonucleotide and/or said second adapter oligonucleotide.

36. The method of claim 35, wherein said sequencing primer comprises SEQ ID NO: 1 or SEQ ID NO: 2.

37. The method of claims 1 or 8, wherein said sequencing comprises a calibration step, wherein said calibration is based on each of the nucleotides at one or more nucleotide positions in said barcode sequences.

38. The method of claims 1 or 5, wherein each of said samples comprises less than 500ng of nucleic acid.

39. The method of claims 1 or 5, wherein said plurality of barcode sequences includes sequences selected from the group consisting of: AAA, TTT, CCC, and GGG.

40. The method of claims 1 or 5, wherein said plurality of barcode sequences includes sequences selected from the group consisting of: AAAA, CTGC, GCTG, TGCT, ACCC, CGTA, GAGT, TTAG, AGGG, CCAT, GTCA, TATC, ATTT, CACG, GGAC, and TCGA.

41. The method of claims 1 or 5, wherein said plurality of barcode sequences includes sequences selected from the group consisting of: AAAAA, AACCC, AAGGG, AATTT, ACACG, ACCAT, ACGTA, ACTGC, AGAGT, AGCTG, AGGAC, AGTCA, ATATC, ATCGA, ATGCT, ATTAG, CAACT, CACAG, CAGTC, CATGA, CCAAC, CCCCA, CCGGT, CCTTG, CGATA, CGCGC, CGGCG, CGTAT, CTAGG, CTCTT, CTGAA, CTTCC, GAAGC, GACTA, GAGAT, GATCG, GCATT, GCCGG, GCGCC, GCTAA, GGAAG, GGCCT, GGGGA, GGTTT, GTACA,

GTCAC, GTGTG, GTTTT, TAATG, TACGT, TAGCA, TATAC, TCAGA, TCCTC, TCGAG, TCTCT, TGACC, TGCAA, TGGTT, TGTGG, TTAAT, TTCCG, TTGGC, and TTTTA.

42. A composition configured for multiplex sequencing comprising: a plurality of target polynucleotides, each target polynucleotide comprising one or more barcode sequences selected from a plurality of barcode sequences, wherein said target polynucleotides are from two or more different samples, and further wherein the sample from which each of said polynucleotides is derived can be identified in a combined sequencing reaction with an accuracy of at least 95% based on a single barcode contained in the sequence of said target polynucleotide.

43. The composition of claim 42, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions.

44. A composition useful in the generation of adapter-tagged target polynucleotides, the composition comprising a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions.

45. The composition of claim 44, further comprising a plurality of second adapter oligonucleotides.

46. The composition of claims 42 or 44, wherein said target polynucleotides are contained in a flow cell.

47. The composition of claims 44 or 45, wherein one or more of said adapter oligonucleotides comprises SEQ ID NO: 1.

48. The composition of claims 44 or 45, wherein one or more of said adapter oligonucleotides comprises SEQ ID NO: 2.

49. The composition of claims 44 or 45, wherein one or more of said adapter oligonucleotides comprise a hairpin structure.

50. The composition of claims 44 or 45, wherein one or more of said adapter oligonucleotides comprise an oligonucleotide duplex.

51. The composition of claims 42 or 44, wherein said barcode sequences are at least 3 nucleotides in length.

52. The composition of claim 44, wherein said first adapter oligonucleotides are grouped in multiples of four such that all four bases are evenly represented at each position along each barcode.

53. The composition of claim 45, wherein each of said second adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions.

54. The composition of claim 53, wherein pairs of said first and second adapter oligonucleotides comprise the same barcode sequence.

55. The composition of claim 53, wherein pairs of said first and second adapter oligonucleotides comprise different barcode sequences.

56. The composition of claims 49 or 50, further comprising a first primer and a second primer, wherein said first primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of said first adapter oligonucleotides, and further wherein said second primers comprise a sequence that is hybridizable to at least a portion of the complement of one or more of said second adapter oligonucleotides.

57. The composition of claim 56, wherein one of said primers comprises SEQ ID NO: 1.

58. The composition of claim 56, wherein one of said primers comprises SEQ ID NO: 2.

59. The composition of claim 49 or 50, further comprising a sequencing primer comprising a sequence hybridizable to at least a portion of the complement of said first adapter oligonucleotide and/or said second adapter oligonucleotide.

60. The composition of claims 42 or 44, wherein said plurality of barcode sequences includes sequences selected from the group consisting of: AAA, TTT, CCC, and GGG.

61. The composition of claims 42 or 44, wherein said plurality of barcode sequences include sequences selected from the group consisting of: AAAA, CTGC, GCTG, TGCT, ACCC, CGTA, GAGT, TTAG, AGGG, CCAT, GTCA, TATC, ATTT, CACG, GGAC, and TCGA.

62. The composition of claims 42 or 44, wherein said plurality of barcode sequences include sequences selected from the group consisting of: AAAAA, AACCC, AAGGG, AATTT, ACACG, ACCAT, ACGTA, ACTGC, AGAGT, AGCTG, AGGAC, AGTCA, ATATC, ATCGA, ATGCT, ATTAG, CAACT, CACAG, CAGTC, CATGA, CCAAC, CCCCA, CCGGT, CCTTG, CGATA, CGCGC, CGGCG, CGTAT, CTAGG, CTCTT, CTGAA, CTTCC, GAAGC, GACTA, GAGAT, GATCG, GCATT, GCCGG, GCGCC, GCTAA, GGAAG, GGCCT, GGGGA, GTTTC, GTACA, GTCAC, GTGTG, GTTTT, TAATG, TACGT, TAGCA, TATAC, TCAGA, TCCTC, TCGAG, TCTCT, TGACC, TGCAA, TGGTT, TGTGG, TTAAT, TTCCG, TTGGC, and TTTTA.

63. A kit useful in the generation of adapter-tagged target polynucleotides, the kit comprising a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions, and instructions for using the same.

64. The kit of claim 63, further comprising a plurality of second adapter oligonucleotides.

65. The kit of claims 63 or 64, wherein one or more of said adapter oligonucleotides comprises SEQ ID NO: 1.

66. The kit of claims 63 or 64, wherein one or more of said adapter oligonucleotides comprises SEQ ID NO: 2.

67. The kit of claims 63 or 64, wherein one or more of said adapter oligonucleotides comprise a hairpin structure.
68. The kit of claims 63 or 64, wherein one or more of said adapter oligonucleotides comprise an oligonucleotide duplex.
69. The kit of claim 63, wherein said barcode sequences are at least 3 nucleotides in length.
70. The kit of claim 63, wherein said first adapter oligonucleotides are grouped in multiples of four such that all four bases are evenly represented at each position along each barcode.
71. The kit of claim 64, wherein each of said second adapter oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions.
72. The kit of claim 71, wherein pairs of said first and second adapter oligonucleotides comprise the same barcode sequence.
73. The kit of claim 71, wherein pairs of said first and second adapter oligonucleotides comprise different barcode sequences.
74. The kit of claims 67 or 68, further comprising a first primer and a second primer, wherein said first primer comprises a sequence that is hybridizable to at least a portion of the complement of one or more of said first adapter oligonucleotides, and further wherein said second primers comprise a sequence that is hybridizable to at least a portion of the complement of one or more of said second adapter oligonucleotides.
75. The kit of claim 74, wherein one of said primers comprises SEQ ID NO: 1.
76. The kit of claim 74, wherein one of said primers comprises SEQ ID NO: 2.
77. The kit of claims 67 or 68, further comprising a sequencing primer comprising a sequence hybridizable to at least a portion of the complement of said first adapter oligonucleotide and/or said second adapter oligonucleotide.
78. The kit of claim 77, wherein said sequencing primer comprises SEQ ID NO:1 or SEQ ID NO: 2.
79. The kit of claim 63, further comprising one or more of: (a) a DNA ligase, (b) a DNA-dependent DNA polymerase, (c) an RNA-dependent DNA polymerase, (d) random primers, (e) primers comprising at least 4 thymidines at the 3' end, (f) a DNA endonuclease, (g) a DNA-dependent DNA polymerase having 3' to 5' exonuclease activity, (h) a plurality of primers, each primer having one of a plurality of selected sequences, (i) a DNA kinase, (j) a DNA exonuclease, (k) magnetic beads, (l) an enzyme comprising RNase H activity, (m) an RNA ligase, and (n) one or more buffers suitable for one or more of the elements contained in said kit.
80. The kit of claim 63, wherein said plurality of barcode sequences includes sequences selected from the group consisting of: AAA, TTT, CCC, and GGG.

81. The kit of claim 63, wherein said plurality of barcode sequences include sequences selected from the group consisting of: AAAA, CTGC, GCTG, TGCT, ACCC, CGTA, GAGT, TTAG, AGGG, CCAT, GTCA, TATC, ATTT, CACG, GGAC, and TCGA.

82. The kit of claim 63, wherein said plurality of barcode sequences include sequences selected from the group consisting of: AAAAA, AACCC, AAGGG, AATTT, ACACG, ACCAT, ACGTA, ACTGC, AGAGT, AGCTG, AGGAC, AGTCA, ATATC, ATCGA, ATGCT, ATTAG, CAACT, CACAG, CAGTC, CATGA, CCAAC, CCCCA, CCGGT, CCTTG, CGATA, CGCGC, CGGCG, CGTAT, CTAGG, CTCCT, CTGAA, CTTCC, GAAGC, GACTA, GAGAT, GATCG, GCATT, GCCGG, GCGCC, GCTAA, GGAAG, GGCCT, GGGGA, GGTTC, GTACA, GTCAC, GTGTG, GTTTT, TAATG, TACGT, TAGCA, TATAC, TCAGA, TCCTC, TCGAG, TCTCT, TGACC, TGCAA, TGGTT, TGTGG, TTAAT, TTCCG, TTGGC, and TTTTA.

83. A method of producing adapter-tagged target polynucleotides, the method comprising:

a) providing a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises a 5' end comprising sequence A and a 3' end comprising sequence A', and further wherein A is hybridizable to A', one of A or A' comprises DNA, and the other of A or A' comprises RNA and 5 or more terminal DNA nucleotides; and,

b) joining at least one of said first adapter oligonucleotides to at least one of said target polynucleotides.

84. The method of claim 83, further comprising the step of cleaving RNA with an enzyme that cleaves RNA from an RNA-DNA heteroduplex.

85. The method of claim 84, further comprising performing the step of extending one or more 3' ends of said target polynucleotides, using said one or more joined adapter oligonucleotides as template.

86. The method of claim 83, further comprising joining at least one of a plurality of second adapter oligonucleotides to said target polynucleotides of each of said samples from step (b), such that at least one of said target polynucleotides comprises said first adapter oligonucleotide at one end and said second adapter oligonucleotide at the other end.

87. The method of claim 86, wherein each of said second adapter oligonucleotides comprises a 5' end comprising sequence B and a 3' end comprising sequence B', and further wherein B is hybridizable to B', one of B or B' comprises DNA, and the other of B or B' comprises RNA and 5 or more terminal DNA nucleotides.

88. The method of claim 83, wherein each of said first adapter oligonucleotides comprises a barcode sequence.

89. A composition useful in the generation of adapter-tagged target polynucleotides, the composition comprising a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises a 5' end comprising sequence A and a 3' end comprising sequence A', and

further wherein A is hybridizable to A', one of A or A' comprises DNA, and the other of A or A' comprises RNA and 5 or more terminal DNA nucleotides.

90. The composition of claim 89, further comprising a plurality of second adapter oligonucleotides, wherein each of said second adapter oligonucleotides comprises a 5' end comprising sequence B and a 3' end comprising sequence B', and further wherein B is hybridizable to B', one of B or B' comprises DNA, and the other of B or B' comprises RNA and 5 or more terminal DNA nucleotides.

91. A kit useful in the generation of adapter-tagged target polynucleotides, the kit comprising a plurality of first adapter oligonucleotides, wherein each of said first adapter oligonucleotides comprises a 5' end comprising sequence A and a 3' end comprising sequence A', and further wherein A is hybridizable to A', one of A or A' comprises DNA, and the other of A or A' comprises RNA and 5 or more terminal DNA nucleotides.

92. The kit of claim 91, further comprising a plurality of second adapter oligonucleotides, wherein each of said second adapter oligonucleotides comprises a 5' end comprising sequence B and a 3' end comprising sequence B', and further wherein B is hybridizable to B', one of B or B' comprises DNA, and the other of B or B' comprises RNA and 5 or more terminal DNA nucleotides.

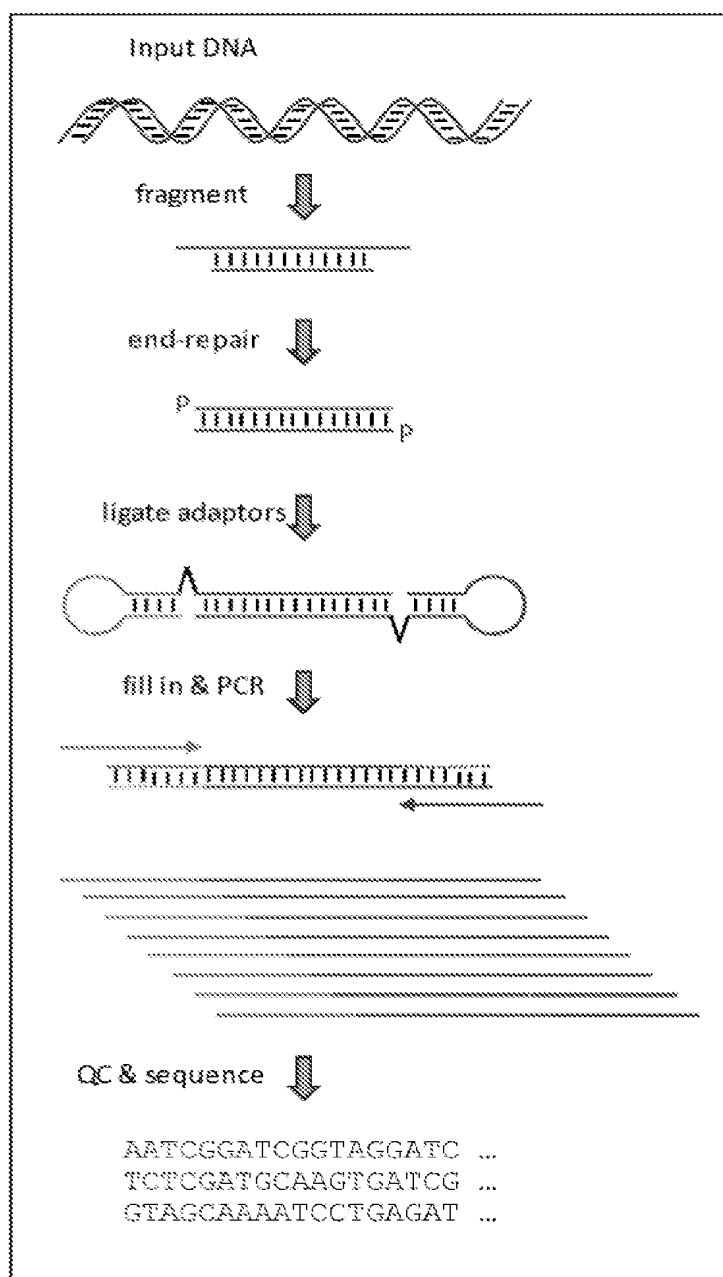


Figure 1

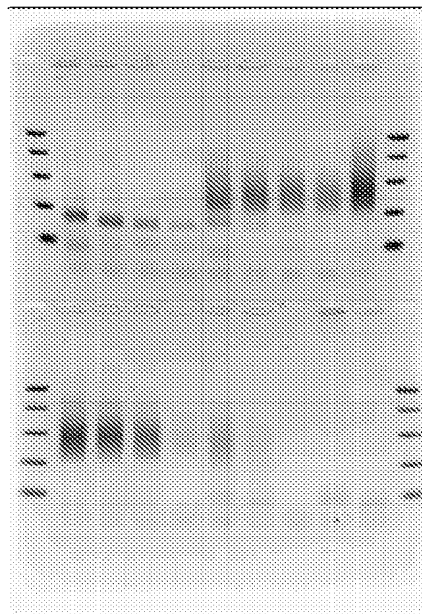


Figure 2A

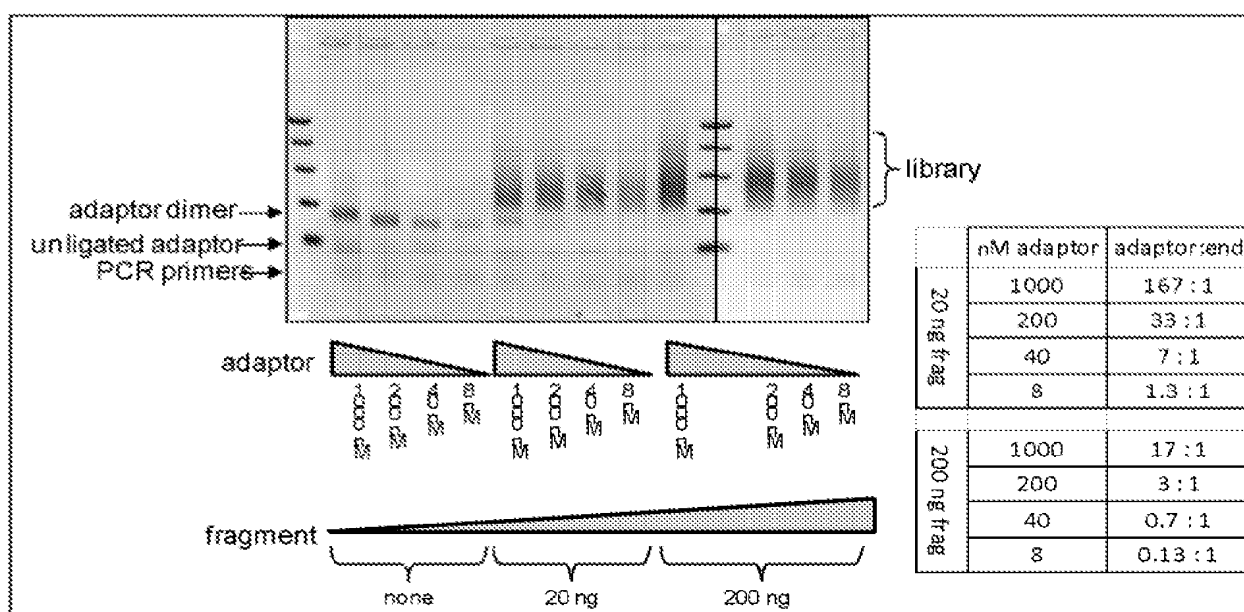


Figure 2B

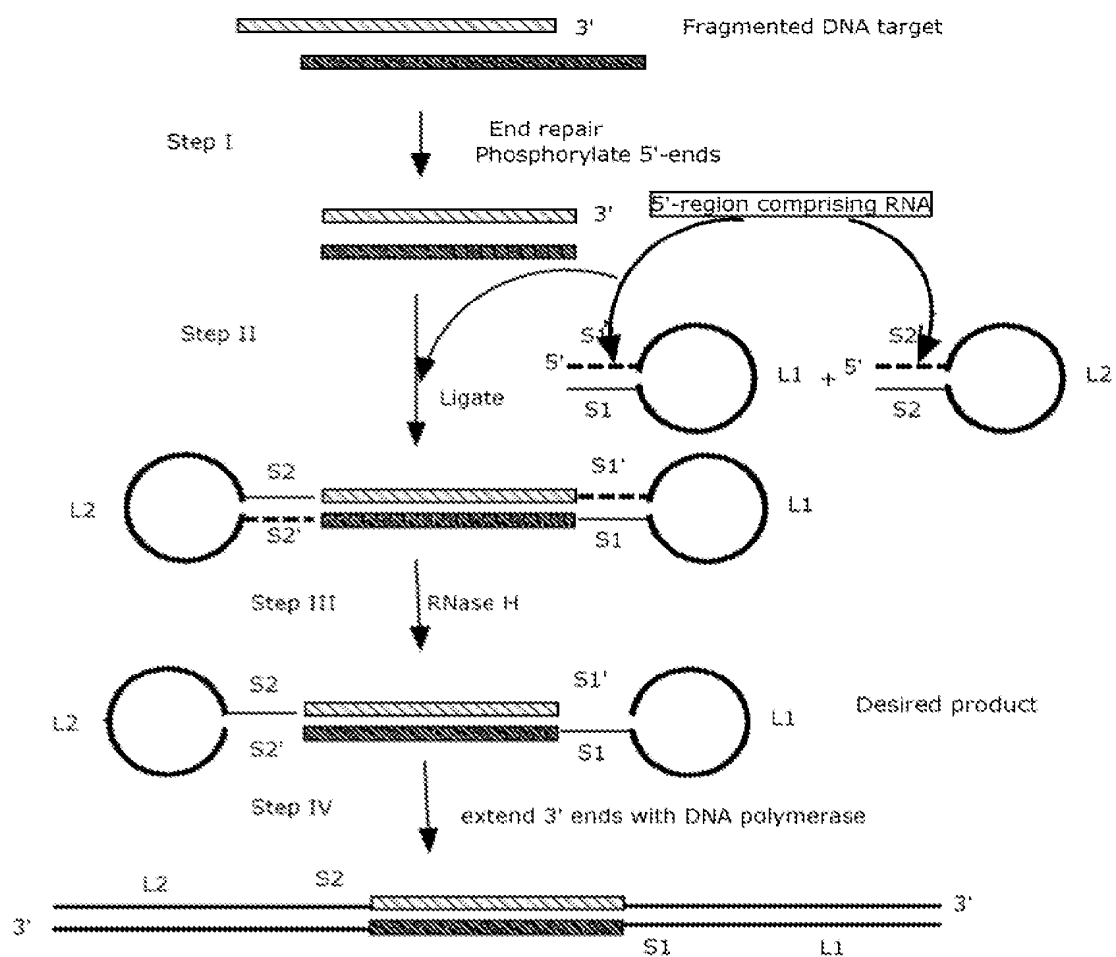


Figure 3

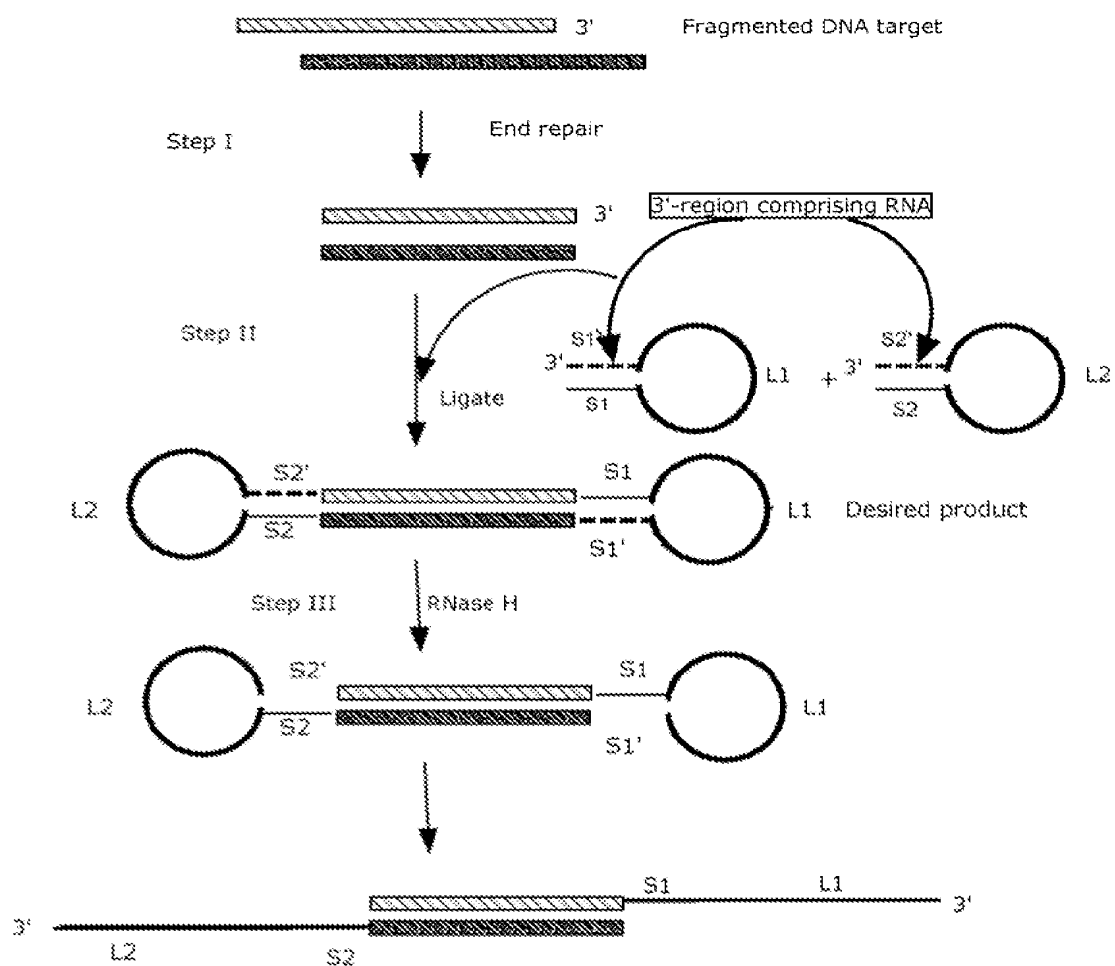


Figure 4

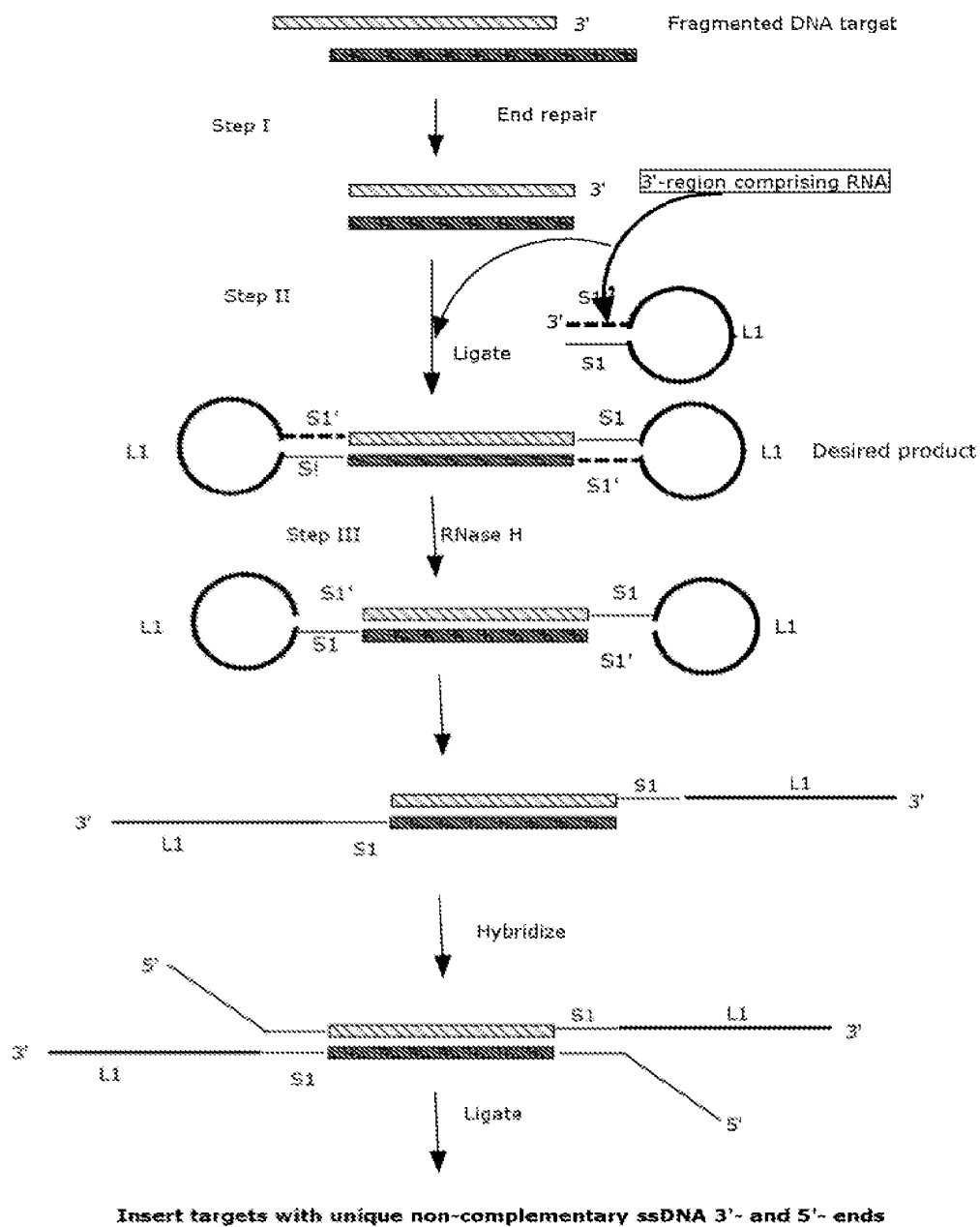


Figure 5

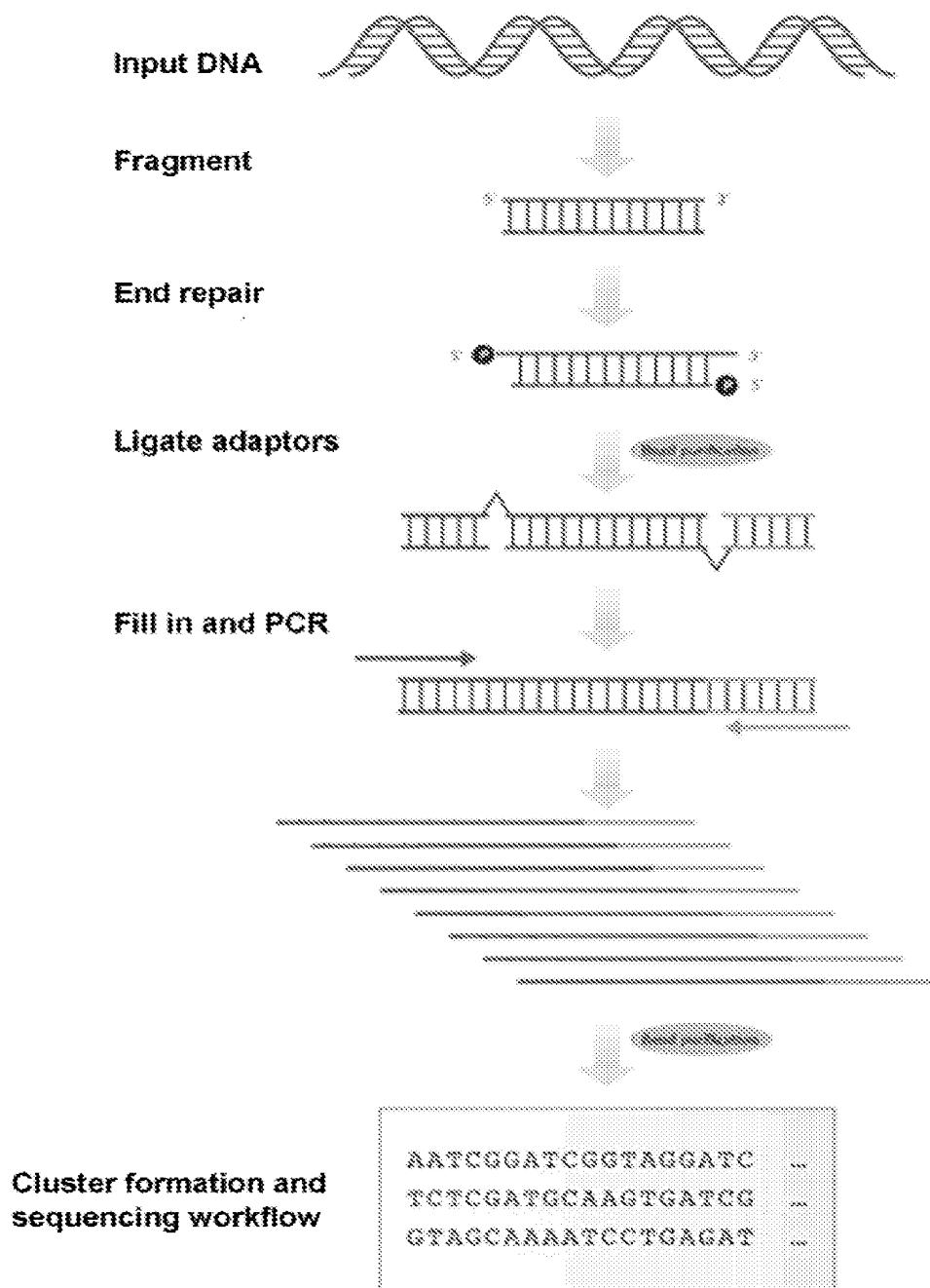
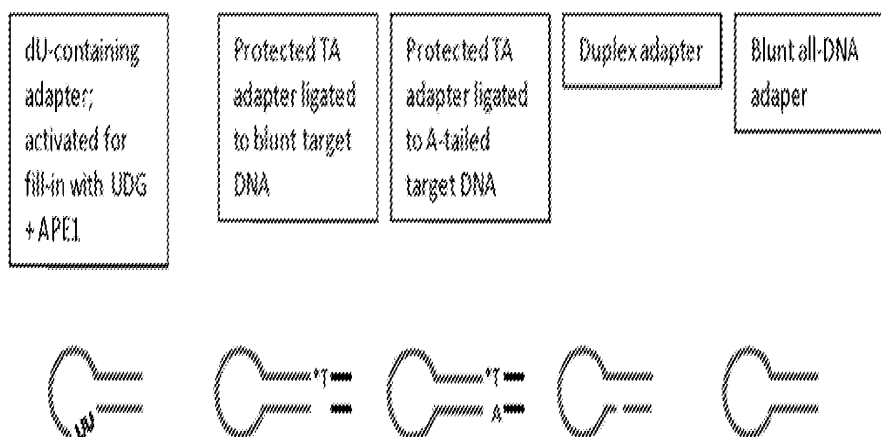


Figure 6



Ligation efficiency: percent of input fragment converted into sequence-able molecules

0.48%	0.0035%	0.20%	0.22%	0.22%
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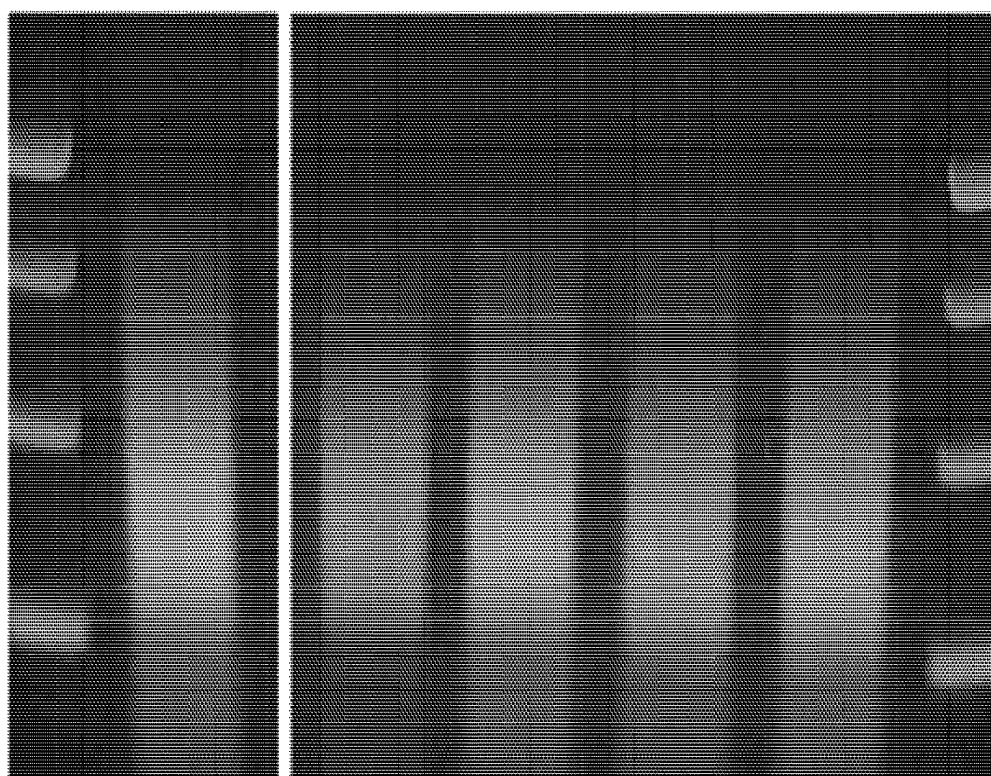


Figure 7

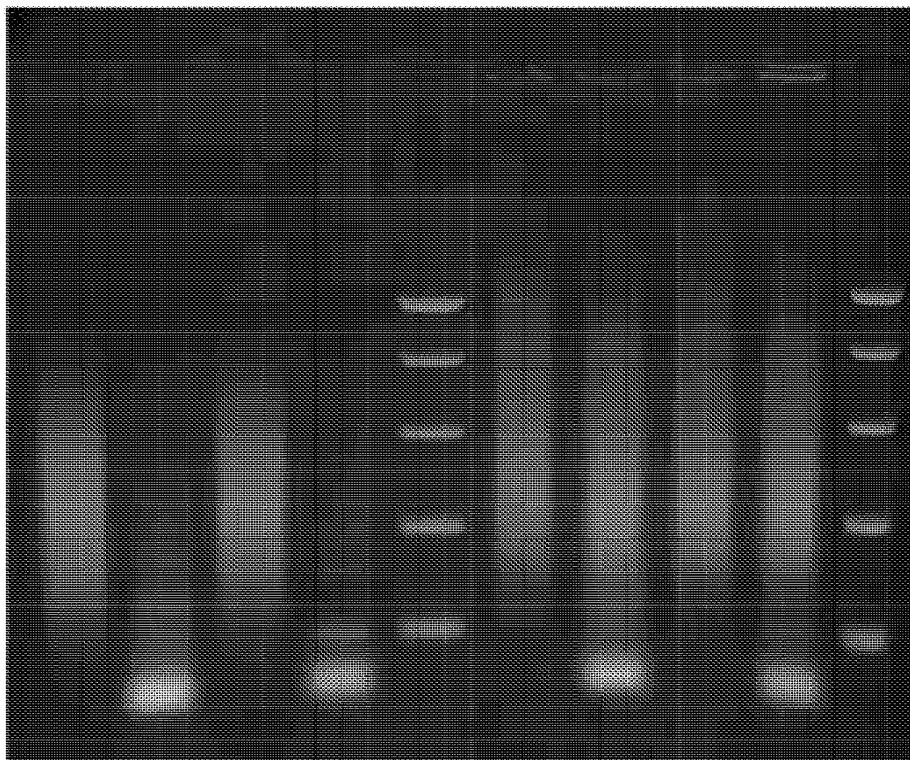


Figure 8

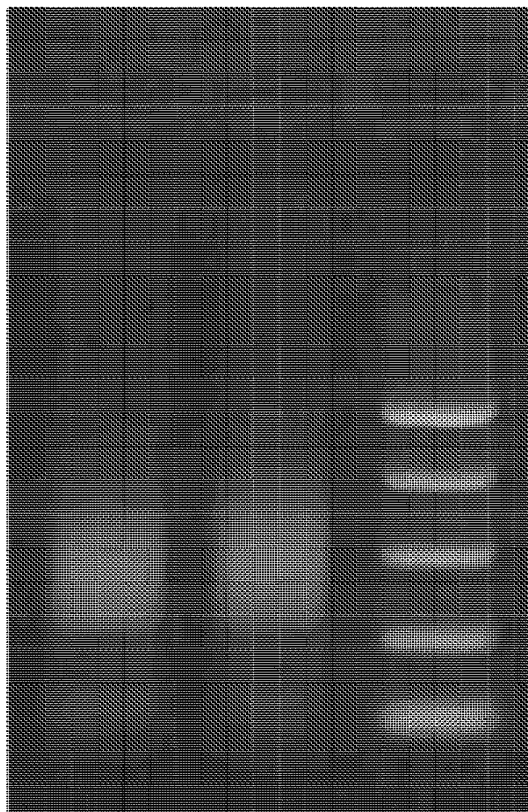


Figure 9

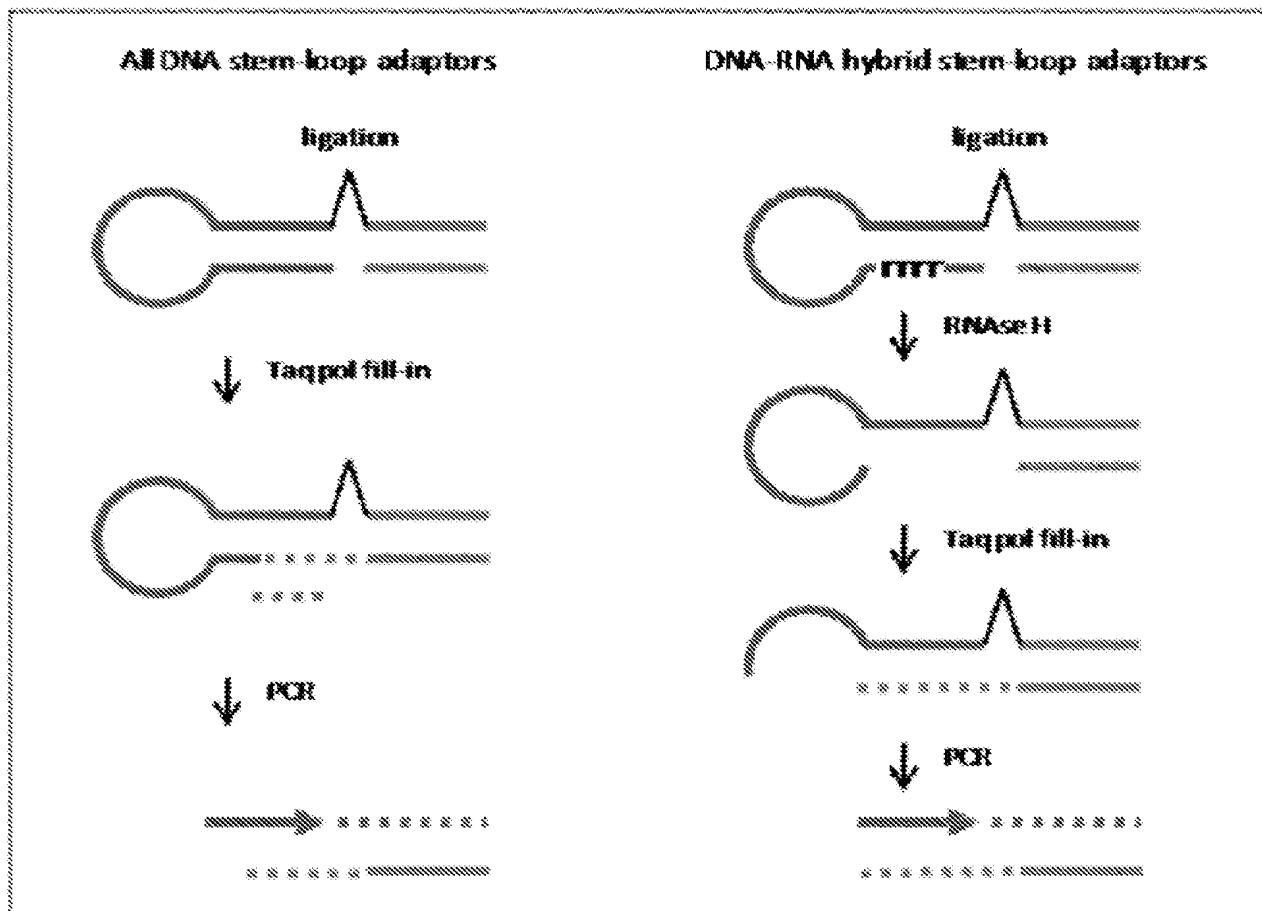


Figure 10



Espacenet

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PROBES FOR SPECIFIC ANALYSIS OF NUCLEIC ACIDS

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The present invention provides a method for detecting or enriching for a target deoxyribonucleic acid (DNA) present in a nucleic acid sample, said method comprising: (a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target DNA; (b) rendering said fragments, including said target fragment, at least partially single-stranded, wherein the single- stranded portion includes an end portion and wherein the length of said single- stranded portion is sufficient to allow hybridisation of at least part of the single- stranded portion of said target fragment to the probe of step (c); (c) contacting the at least partially single-stranded fragments of step (b) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein: (i) oligonucleotide A is a single- stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part of said single- stranded portion of said target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including

one end, of oligonucleotide B of the probe, and (ii) oligonucleotide B is a single-stranded oligonucleotide which may contain or carry at least one element for detection and/or enrichment of said target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A, such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment; (d) ligating oligonucleotide B of said probe to the part of the single-stranded portion of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid; and (e) detecting or enriching for said probe-target fragment hybrid. Kits for use in the method of the invention are also provided.

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(54) Title: PROBES FOR SPECIFIC ANALYSIS OF NUCLEIC ACIDS

(57) Abstract: The present invention provides a method for detecting or enriching for a target deoxyribonucleic acid (DNA) present in a nucleic acid sample, said method comprising: (a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target DNA; (b) rendering said fragments, including said target fragment, at least partially single-stranded, wherein the single-stranded portion includes an end portion and wherein the length of said single-stranded portion is sufficient to allow hybridisation of at least part of the single-stranded portion of said target fragment to the probe of step (c); (c) contacting the at least partially single-stranded fragments of step (b) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein: (i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part of said single-stranded portion of said target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and (ii) oligonucleotide B is a single-stranded oligonucleotide which may contain or carry at least one element for detection and/or enrichment of said target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A, such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment; (d) ligating oligonucleotide B of said probe to the part of the single-stranded portion of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid; and (e) detecting or enriching for said probe-target fragment hybrid. Kits for use in the method of the invention are also provided.

WO 2011/009941 A1

Probes for specific analysis of nucleic acids

The present invention relates to a method for detecting or enriching target nucleic acids in a complex nucleic acid sample. In such a method the sample is fragmented and fragments containing the target nucleic acid are covalently attached, at or near one end by nucleic acid ligation ("single-sided ligation", i.e. one end of the target fragment is not involved in the ligation reaction and remains free), to a single target-specific probe per target fragment. Optionally, common nucleic acid adaptors may be non-target-specifically added to said free end of the fragments. The probes may contain elements facilitating enrichment of the target fragments, such as by purification (e.g., by immobilisation to a solid phase), or amplification (e.g., by utilising elements in the probes and optional adaptors). In this way, target nucleic acids may be obtained from complex samples for subsequent analysis by, e.g., nucleic acid sequencing, microarray, qPCR, visualisable hybridisation probes, *in situ* analyses, etc. The feature of the present invention of providing (and using) a ligation probe specific for only one end of a target nucleic acid (or target nucleic acid fragment) is advantageous, and facilitates particular applications not possible with known methods.

It is often desired to isolate large numbers of sub-genomic sequences to allow their further characterisation, e.g. the isolation of the genomic regions believed to be of relevance to a particular physiological or pathological condition. This is particularly so following the advent of parallel methods of high-throughput sequencing, which necessitate methods for rapidly isolating genomic sequences of interest for use in such sequencing methods.

One known method for amplifying (amplification constituting a means of "enriching" according to the present invention) in parallel a plurality of target nucleic acids from a sample of nucleic acids is disclosed in WO 2005/111236. In this method, partially double-stranded "Selector" nucleic acid molecules (either a single symmetrical molecule in which the longer strand overhangs at both ends, or two asymmetrical molecules each having a single-stranded overhang at only one end) are hybridised via their single-stranded overhangs in a target-specific manner to both ends of single-stranded (denatured) target fragments resulting from fragmentation of the nucleic acid sample (in the case of the single symmetrical Selector, the hybridised Selector-target fragment becomes circularised). In a particular embodiment of the method using the symmetrical Selector, only one end of the target fragment hybridises to an end of the Selector, the other end of the Selector

hybridising internally of the target nucleic acid fragment and requiring a structure-specific endonuclease to resolve the resulting structure by cleaving off the portion of the target fragment "protruding" beyond the internal hybridised region. In all cases, therefore, the amplifiable portion of the target fragment is delineated by the regions (whether both end regions or one end and one internal region) of known sequence to which the Selector(s) has been designed to hybridise. Following hybridisation (and, where appropriate, resolution of the secondary structure) the Selector(s) and target nucleic acid fragment are joined by ligation to give (i) in the case of the symmetrical Selector, a circular nucleic acid molecule and (ii) in the case of the asymmetrical Selectors a linear molecule comprising the target fragment flanked by Selectors. The double-stranded region of the Selector(s) contains a primer pair motif which is common to the plurality of different target-specific Selectors used in a multiplex assay. Hence, amplification of multiple target fragments can be achieved simultaneously whilst avoiding amplification artefacts which can result from the use of multiple, different primer pairs.

In the method of WO 2005/111236, two target-specific hybridisation events are required for each target fragment; hybridisation of Selector(s) to both ends, or to one end and one internal sequence, of the target fragment. This inevitably requires knowledge of the sequence of the target nucleic acid at least at these two hybridising regions, in order for target-specific hybridisation probes to be designed.

However, it is often desired to isolate or enrich for genomic sequences or fragments based on knowledge of only one region of sequence, i.e. wherein the fragment (or region of sequence) it is desired to isolate is not bounded at both ends (delineated) by regions of known sequence. This will frequently be the case when the need to isolate the target sequence (e.g. fragment) arises from a desire to subject the sequence/fragment to sequencing, i.e. at least part of the sequence is not known. A particular example of this is the analysis of chromosomal translocation breakpoints, where it is desired to determine the precise sequence of the region of a chromosome to which a region of another chromosome, containing known sequence, has become fused. Other examples include the analysis of splicing patterns or VDJ recombination events. Hence, there is a need for a method of rapidly and efficiently detecting (or enriching for or capturing) one or a plurality of target nucleic acids which does not require knowledge of the sequence of regions at both ends of the corresponding target nucleic acid fragment.

It has now been found that the limitation of the above-described Selector(s) method may be overcome through the use of a single (i.e. a single species of) probe, that is a single (or single species of) probe per target nucleic acid, which has

specificity to a region of known sequence in the nucleic acid fragment corresponding to the target nucleic acid. In other words, the present invention is based on the novel feature of using only one target-specific probe for each target nucleic acid, which binds in a target-specific manner only once (more particularly at only one site) in the target nucleic acid fragment. Thus, for each target nucleic acid fragment resulting from fragmentation of the nucleic acid sample, only one target-specific hybridisation and ligation ("binding") event occurs; the target-specific ligation is single-sided with respect to the target nucleic acid fragment and therefore only a single region of known sequence in the target nucleic acid fragment is required in order to design the probe. In other words, there need only be, and is only, one defined binding site for the probe in each target nucleic acid fragment, i.e. the sequence of the target fragment needs to be defined at only one site, e.g. at only one end. The method of the invention thereby enables and, advantageously, facilitates the detection or enrichment of genomic nucleic acids lacking known sequence at both ends (i.e. having known sequence at only one end).

The use of such a single target-specific probe offers further advantages beyond the ability to detect or enrich for target nucleic acids having unknown sequence at one end (i.e. the end other than that to which the target-specific probe hybridises). The ligation of the probe to the target nucleic acid fragment results in a covalent connection between these molecules. Thus, the actual genomic fragment, and not an amplification product thereof, becomes covalently connected to the probe. In certain embodiments of the invention the probe contains elements facilitating immobilisation of the probe to a solid phase. In such embodiments the covalent connection between the target nucleic acid fragment and the probe results in a stable capture of the former and allows the use of highly stringent washing steps to remove non-specifically hybridised (non-ligated) fragments, resulting in a high specificity. Washes of such high stringency cannot be used with known hybridisation- (as opposed to ligation-) based methods such as microarray capture and fluorescence *in situ* hybridisation (FISH), and the ability to use such washes in the present method is therefore advantageous.

The use of the single target-specific probes in the methods of the invention need not be limited only to methods using genomic DNA. In view of the favourable results obtained using human genomic DNA (see the Examples) it is envisaged that the methods of the invention would work equally well with any DNA sample that contains one or more target nucleic acids. Thus, for example, the methods of the invention could use one or more cDNA samples, e.g. cDNA obtained, derived or synthesised from the RNA of whole organisms, specific tissues, cell types etc or

biological samples exposed to various conditions which may alter the gene expression of said sample resulting in an altered cDNA population.

Accordingly, the present invention provides a method for detecting or enriching for a target deoxyribonucleic acid (DNA) present in a nucleic acid sample, said method comprising:

(a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target DNA;

(b) rendering said fragments, including said target fragment, at least partially single-stranded, wherein the single-stranded portion includes an end portion and wherein the length of said single-stranded portion is sufficient to allow hybridisation of at least part of the single-stranded portion of said target fragment to the probe of step (c);

(c) contacting the at least partially single-stranded fragments of step (b) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:

(i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part of said single-stranded portion of said target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and

(ii) oligonucleotide B is a single-stranded oligonucleotide which may contain or carry at least one element for detection and/or enrichment (in particular detection, amplification and/or capture) of said target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A, such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment;

(d) directly or indirectly ligating oligonucleotide B of said probe to the part of the single-stranded portion of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid; and

(e) detecting or enriching for said probe-target fragment hybrid.

As described in more detail below, the method of the invention may advantageously include an additional step, between steps (a) and (b) in which a common nucleic acid adaptor is non-target-specifically annealed to the ends of the fragments. Such a method involving the use of a common adaptor represents a preferred embodiment of the present invention. In particular, in such a step the

annealed adaptor becomes ligated to the fragments only at the 3' ends or only at the 5' ends of the strands of the fragments. More particularly, the adaptor is ligated to one end of the strand of the target fragment to which, at the other end, oligonucleotide B of the probe is ligated in step (d). In other words, in the target fragment the adaptor is ligated at the opposite end of the strand to which the target-specific probe becomes ligated.

The method of the invention may be performed as described above ("simplex" format) to enrich for a single (i.e. a single species of, which will normally be present in many copies) target fragment or for a plurality of target fragments which are sufficiently similar in sequence so as to be possible to enrich for using the same probe. In this context it will be seen that the term "single" as used in relation to the target-specific probe in part (c) means single in the context of a particular target fragment, namely that one probe (or more particularly one type or species of probe) is used for each target fragment (i.e. a single probe per target fragment). Thus, where there is only one target fragment only one probe (in the sense of one species of probe) will be used. Accordingly, part (c) above may alternatively be expressed as:

"(c) contacting the at least partially single-stranded fragments of (b) with oligonucleotides A and B of a target-specific nucleic acid probe wherein for each target fragment a single probe is used, wherein"

It is clear from the above that "single" probe means single species of probe and does not imply any limitation on the actual number of probe molecules used.

Alternatively, a plurality (i.e. a plurality of species) of probes may be used in a "multiplex" format simultaneously to enrich for a plurality of target DNAs. Hence, in such a latter aspect the method as defined above is for enriching for a plurality of target DNAs, wherein in step (c) said target fragments are contacted with oligonucleotides A and B of a plurality of nucleic acid probes, each having an oligonucleotide A with a different first target-specific part, whereby a plurality of different target fragments may be annealed to said probes. In such a multiplex method, as stated above, for each target fragment of the plurality (i.e. each different type or species of target fragment) a single (i.e. in the sense of a single species of) probe will be used. Thus, a plurality of probes will be used, with a (different) single probe for each target fragment. The term "plurality" as used herein means 2 or more (or at least 2), more particularly 3 or more (or at least 3), or 4, 5, 6, 8, 10, 15, 20, 30, 50, 70 or 100 or more etc. In certain embodiments even higher numbers of probes may be used and very many different targets may be enriched or detected, e.g. 500, 1000, 2000, 5000 or 10,000 or more. For example, 10, 100, 1000 or 10000 different

probes may simultaneously be used to detect or enrich for, respectively, 10, 100, 1000 or 10000 different target fragments.

The term "enriching" as used herein is used broadly and includes any means of selecting, isolating and/or capturing a DNA sequence of interest ("target DNA") from a nucleic acid sample which contains other nucleic acids, particularly other DNAs, in addition to (and including DNAs being part of the same DNA molecule as) the target DNA. Thus "enriching" encompasses any means of practically, if not necessarily physically, "separating" the target DNA from the other nucleic acids present in the sample, more particularly by means of covalently attaching a probe thereto in order that the target DNA may be subjected to analytical and/or preparative or synthetic, or other enrichment, techniques. "Enriching" for the target DNA as used herein may, for example, be amplification by one of the many known methods of nucleic acid amplification, or may be physical capture, for example by immobilisation to a solid phase, optionally followed by amplification.

Although as discussed further below, in certain embodiments the method may involve immobilisation on a solid phase, this is not a necessary feature of the method. Thus, the method may be carried out in solution, i.e. may be a homogeneous method.

The term "detecting" is also used broadly herein and includes any means of detecting or determining or assaying for the presence of the target DNA, or any means of analysing the target DNA. Direct analysis of the target DNA sequence (i.e. sequencing of all or any part of the target DNA) is encompassed by the term detecting. As described further below this may be accomplished by any means enabled by the probe ligated to the target molecule, such as the presence in the probe of a sequencing primer binding site. Thus, the method of the invention allows a selected target fragment to be sequenced. More particularly, a target fragment selected by means of the target-specific probe according to the present invention may be directly sequenced. Of course, the invention also covers indirect analysis, e.g. sequencing, of the target DNA, for example analysis, e.g. sequencing, of an amplified target DNA, or of a captured target DNA fragment.

The step of detecting or enriching for the target DNA involves detecting or enriching for the probe-target fragment hybrid. Thus, detection or enrichment of the target DNA may be achieved by any means of detection or enrichment which is selective for the hybrid. As described in more detail below, this may be achieved by means of detection and/or enrichment (e.g. capture and/or amplification) elements which are present in the probe (and thereby incorporated into the hybrid, but not present on other, non-target, fragments present in the sample which have not

become ligated to the probe) or more generally by any means which is dependent on the ligation of the probe to the target fragment or, put another way, dependent on formation of the probe-target fragment hybrid.

The term "target DNA" as used herein means a DNA of interest which it is desired to detect or for which it is desired to enrich. This will generally be a part (or a portion or segment) of a longer DNA molecule which may be present in the sample. Thus, it may be a region or stretch of a longer DNA present in the nucleic acid sample (or more particularly, DNA sample). The target DNA may be of any length, but in order to be detected or enriched by the method of the invention must comprise or be contained within a fragment (the "target fragment") produced by the step of fragmenting the nucleic acid sample. The sequence of the target DNA may not be known, providing that at least a portion of the target fragment is of known sequence in order to facilitate the design of the probe, which must be able to hybridise to a single region of the target fragment.

Whilst the target DNA may be of any length, advantageously the methods disclosed herein may be used for detecting or enriching large target fragments or, alternatively viewed, target fragment polynucleotides of a minimum length, by which is meant target fragments comprising at least 30 nucleotides. More preferably, the target fragments comprise at least 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 250 or 300 nucleotides. Where the target fragment comprises an overhang, e.g. as a result of cleavage (fragmentation), the minimum length of the target may include or exclude the overhang sequence.

The "nucleic acid sample" referred to above may be any sample which contains any amount of nucleic acid, from any source or of any origin, in or from which it is desired to detect or enrich a target DNA known or suspected to be comprised therein. More particularly, the sample may be any sample that contains DNA. The sample may be complex, e.g. whole genomic DNA, cDNA from a whole organism, tissue or cell population, or a fraction thereof. In this regard it may for example be a direct product of a nucleic acid isolation procedure, or of a cell lysis procedure, or it may be further be fractionated or purified in some way, e.g. it may contain nucleic acids which have been partially or fully separated in some way, or treated in any way, e.g. RNA treated to produce cDNA. The sample may be from any eukaryotic or prokaryotic or viral source, e.g. may be of microbial (for example bacterial or fungal), plant, or animal (e.g. vertebrate, mammalian or primate) origin. In a particular aspect, the sample may be of human origin e.g. human genomic DNA or cDNA. The sample may be from a single origin, or may be formed from a pooling of a plurality of samples from different origins. In the latter case, the fragmenting step (a),

and optionally one or more further steps of the method, may be performed on the samples separately prior to said pooling. For example, said nucleic acid sample may represent the pooling of a number of patient samples, such that the method of the invention allows parallel enrichment of target DNAs from multiple patients.

Thus, the target DNA is preferably genomic DNA. It may represent total genomic DNA or it may be a sub-fraction thereof. Thus, the sample may comprise or may be derived from genomic DNA, e.g. by separating/isolating genomic DNA directly or copying (e.g. amplifying) genomic DNA.

Alternatively, the target DNA is preferably cDNA (complementary or copy DNA). The, the sample may comprise cDNA or a subfraction thereof, wherein cDNA is DNA that is complementary to messenger RNA or DNA that has been synthesized from messenger RNA by reverse transcriptase. Preferably, in the context of the present invention the cDNA has been treated to comprise double stranded DNA. Thus cDNA can be considered to be a copy of the RNA present in a cell at the time of extraction or isolation or a fraction thereof, i.e. it represents all or some of the genes that were expressed in said cell at the time of isolation. Hence, a cDNA sample may represent the genes expressed from a whole organism or a part thereof, e.g. a tissue or cell type or group or sub-group thereof, and may further represent genes expressed under specific conditions, e.g. at a particular time, in a specific environment, at a stage of development or in response to stimulus etc. The cDNA may represent a subfraction of the RNA isolated from any of the above sources, e.g. the RNA or cDNA may be fractionated, e.g. by size, to encompass only a proportion of the genes expressed in the source. The cDNA may be derived from a single source or multiple sources as described elsewhere herein. The sample may comprise cDNA derived directly from the mRNA or the cDNA may be derived indirectly, e.g. the cDNA may be amplified, e.g. via the production of a cDNA library.

In a first step (a) of the method the nucleic acid sample known or suspected to contain the target DNA is fragmented to produce fragments, amongst which will exist (if the target DNA is present in the sample, and if the method of fragmentation is selected appropriately) at least one (i.e. at least one species of) fragment containing the target DNA. The term "fragmenting" is used broadly herein to include any means by which the nucleic acid in the sample may be fragmented or cleaved (i.e. divided or "cut" into smaller pieces or fragments). Thus, fragmentation may be carried out enzymatically, e.g. using restriction or other endonucleases or nucleases such as DNase, and/or physically, e.g. by nebulisation or sonication or any shear-based methods. Such physical methods result in unpredictable, non-sequence-specific fragmentation, as do certain (non-restriction) endonucleases. Thus both random, and

pre-determined (or site-specific) fragmentation is encompassed, but the latter is preferred. Accordingly, fragmentation using an enzyme which cleaves at a known or defined site is preferred, in other words enzymes which cleave sequence-specifically or structure-specifically, or, put another way, which cleave to generate ends of known (defined) sequence, for example restriction endonucleases and FLAP endonucleases. However, also encompassed by the reference to "fragmenting" in step (a) is fragmentation of a nucleic acid sample which inherently may occur as a result of the age of a sample, the conditions in which it is stored and any treatment of the sample (e.g. fixation, for example in a tissue sample), and the degradation to which these factors contribute. Any suitable class of restriction endonuclease may be used, including type II and type IIs enzymes. The use of type IIs endonucleases is particularly advantageous as these result in fragments which will not have the same end sequences, thereby improving the chances targeting the probe to the intended target fragment through the use of an oligonucleotide A of appropriate sequence. Alternatively, the cleaving (fragmenting) may be achieved using a flap endonuclease, wherein an added nucleic acid or oligonucleotide is only partially hybridisable, due to being partially double-stranded, to a sequence in the nucleic acid sample resulting in a protruding non-hybridised region of the nucleic acid sample adjacent to a hybridised region. This secondary structure is the substrate for a so-called structure-specific "flap endonuclease" enzyme which cleaves the nucleic acid sample at the junction of the hybridised and non-hybridised regions (Lyamichev V et al, Science. 1993 May 7;260(5109):778-83). The use of a flap endonuclease may be advantageous in the absence of a (known) restriction enzyme recognition sequence near to the target DNA within the nucleic acid sample, as it allows cleavage (fragmentation) to be targeted at any region of known sequence. Flexibility in positioning the site of cleavage is thereby afforded. When using a flap endonuclease, a preceding physical fragmentation step may be desirable.

Fragmenting means may be used in combination, e.g. the use together of two or more endonucleases, more particularly two or more restriction endonucleases, or the use together of an enzymatic and a physical means. Furthermore, the nucleic acid sample may be differently fragmented in separate aliquots, which aliquots are then pooled and together subjected to the remaining steps of the method of the invention.

Hence, the fragmenting may be achieved by separating the nucleic acid sample into a plurality of aliquots and fragmenting the respective aliquots with different means or different combinations of means, such means being for example restriction enzymes. The aliquots are then subjected to the remaining steps of the

method and may be pooled at any point during the method, for example before step (b), before step (c), before step (d) or before or after step (e) to give the (single) nucleic acid sample referred to in the above method. Such an embodiment should be distinguished from the separate fragmenting and subsequent pooling discussed above, in which case the samples are of different origins (rather than aliquots of a single sample). However, in that aspect the samples of different origin may themselves respectively be fragmented in multiple separate aliquots and respectively pooled as mentioned above, before the pooling to give the (single) nucleic acid sample, referred to in the above method, for use in subsequent steps of the method.

Known heterozygous polymorphisms in the sample nucleic acid may be exploited by fragmenting the sample using, if possible, endonucleases which recognise a sequence inactivated in at least one instance by such a polymorphism. By designing probes targeted to the fragments produced in the presence and absence of cleaving at the polymorphic endonuclease recognition site said haplotype-specific fragments may independently be enriched and analysed.

After the fragmenting step the fragments of the nucleic acid sample, including the fragment which contains the target DNA, are rendered at least partially single-stranded (step (b)). Where the fragments are not made completely single-stranded, they will at least be single-stranded at an "end portion", which means that the part of such fragments which is single-stranded will include one end of the fragments, and the single-stranded portion will be of sufficient length to allow, in the case of the target fragment, hybridisation of at least part of said portion to the probe. Thus "rendering at least partially single-stranded" as used herein encompasses all means of causing a double-stranded DNA fragment to become single-stranded wholly or at least at an end-containing portion. Such means include denaturation, e.g. by heat or pH or through the use of chemicals, as is known in the art. Heat denaturation is particularly preferred. Rendering the fragments only partially single-stranded such that they remain largely or at least partially double-stranded is advantageous, especially for the enrichment of long genomic sequences, as this avoids to some extent the undesirable cross-reactivity between single-stranded nucleic acid fragments and thereby reduces the incidence of hybrids which must be distinguished between.

Alternatively, at least partial single-strandedness can be achieved by 3' or 5' exonucleolysis using an appropriate 3' or 5' exonuclease. Starting at a free double-stranded fragment end, such enzymes progressively degrade or digest one strand of a double-stranded nucleic acid, leaving the complementary strand and rendering the nucleic acid single-stranded along the length of the enzyme's action. The extent of

exonucleolytic degradation (i.e. the length of the resulting single-stranded region) may be controlled by the duration of the reaction. The duration of the exonuclease reaction is chosen in order that an appropriate length of one end of the strands of the fragments is removed. The extent of digestion must be sufficient to allow a productive hybridisation (i.e. hybridisation capable of templating ligation) with the first target-specific part of oligonucleotide A of the probe, but not so much that the fragment loses all double-strandedness and becomes two single-stranded fragments. Suitable exonucleases are known in the art and include e.g. exonuclease III (3') and lambda exonuclease (5'). The use of exonuclease III is particularly preferred. As regards the use of exonucleases it should be noted that, if fragmentation is to be performed in step (a) by restriction endonucleolysis, it must be borne in mind when selecting the endonucleases whether they produce 5' or 3' overhanging, or blunt, ends, as certain types of restriction ends are poor substrates for certain exonucleases. The substrate preferences of exonucleases are known in the art. For example, exonuclease III does not favour 3' overhanging ends, and hence the use of endonucleases which leave 5' overhanging ends is in some aspects preferred. As mentioned above, the single-stranded end-containing portion of the target fragment must be able to hybridise, at least in part, to the probe. Thus, the single-stranded portions of the fragments must be of sufficient length to support, in step (c), stable base-pairing between at least part of the single-stranded portion of the target fragment and the first target-specific part of oligonucleotide A of the probe. "Sufficient to allow hybridisation" as used herein therefore means that the partially single-stranded target fragment must be single-stranded to the extent necessary to permit a productive hybridisation with the probe, i.e. a hybridisation capable of templating ligation of the hybridised portion of the fragment to oligonucleotide B of the probe in step (d). This does not require, but does include, 100% complementarity between the first target-specific part of oligonucleotide A of the probe and the corresponding region of the single-stranded portion of the target fragment. "Complementary", as used herein, means functionally complementary, i.e. a level of complementarity sufficient to mediate hybridisation, which encompasses degrees of complementarity less than 100%.

The reference in step (b) above to "at least part" of the single-stranded portion of the target fragment encompasses, for instances where said single-stranded portion is longer than the first target-specific part of oligonucleotide A of the probe to which at least part of said single-stranded portion is complementary, hybridisation of said part of oligonucleotide A to a portion of said single-stranded portion which includes the end of the target fragment as well as, in the alternative, to an internal non-end portion. Thus, the first target-specific part of oligonucleotide A

has functional complementarity, and therefore hybridises, either to an end-containing portion or to an internal non-end-containing portion of the single-stranded portion which, as mentioned above, includes an end portion. Thus, in certain embodiments of the invention the first target-specific part of oligonucleotide A hybridises at (is functionally complementary to) one end of the single-stranded portion of the target fragment. As discussed in more detail below, in certain other embodiments of the invention the hybridisation and ligation steps (c) and (d) occur through resolution of a generated secondary structure using a flap endonuclease (as discussed above in the context of fragmenting step (a)), the substrate for which is generated by hybridisation of oligonucleotide A of the probe to such an internal non-end portion of the target fragment.

Once the fragments of the nucleic acid sample have been rendered at least partially single-stranded in step (b) they are contacted with oligonucleotides A and B of the single target-specific probe of step (c). As noted above, reference to a "single" probe is intended as a reference to a single species of probe; although in practice many copies of the probe (and other nucleic acid reagents) will be used, for a given particular target fragment only one probe (albeit many copies thereof), rather than two or more different probes, is contacted with the fragments of step (b). The probe comprises or consists of oligonucleotides A and B hybridised together. As oligonucleotide A has a first target-specific part which is not complementary to oligonucleotide B, the probe is partially single stranded. As is apparent from step (c), the probe may be provided "intact" (with oligonucleotides A and B pre-hybridised) or oligonucleotides A and B may be added un-hybridised in which case hybridisation to form an intact probe will occur during the step (c) of annealing the probe (oligonucleotide A) with the target fragment (as used herein, "annealed" means non-covalently joined or connected and therefore does not include ligation). Reference in step (c) to "oligonucleotides A and B of a single target-specific nucleic acid probe" also encompasses a probe wherein oligonucleotides A and B are connected together, or joined, directly or indirectly, to form a single contiguous oligonucleotide, oligonucleotides A and B representing a self-complementary portion thereof (wherein oligonucleotides A and B may be annealed (hybridised) together). Thus, in such a probe oligonucleotides A and B may represent the respective "parts" (more particularly "hybridising parts") of a single continuous partially self-complementary oligonucleotide. In such a case, the end of the probe other than the single-stranded target-specific part (of oligonucleotide A) is a hairpin or hairpin loop. Hence, as used herein, reference to "oligonucleotides" A and B is a reference to the respective parts of such an oligonucleotide. Alternatively viewed, oligonucleotides A and B may be

seen as oligonucleotide sequences, which may be provided either as separate oligonucleotide molecules (i.e. separate moieties) or they may be part of a single oligonucleotide molecule. When oligonucleotides A and B are parts of such a single oligonucleotide, the oligonucleotide may be pre-hybridised or un-hybridised when contacted with the at least partially single-stranded fragments in step (c).

Advantageously, the fragments are contacted with un-hybridised oligonucleotides A and B of the probe, with a large amount of oligonucleotide A, and an even greater amount of oligonucleotide B (except in the case that oligonucleotides A and B are parts of a single oligonucleotide), being added in order that as high a proportion as possible of the target fragments are annealed thereto. The use of oligonucleotides A and B of the probe in excess, or large amounts of these probe oligonucleotides relative to the amounts of the fragments of the nucleic acid sample is, in addition to minimising the requirement for sample material, to try to ensure that a large fraction (at or close to 100%) of the available target fragments are bound to the probe, which is necessary for equal representation of the target fragments. Equal representation is important in, for example, sequencing applications, for efficient resequencing. Where oligonucleotides A and B are provided as part of a single probe, this helps to ensure that A and B oligonucleotides are hybridised together without having to add excess oligonucleotides.

The target-specific nucleic acid probe, and hence oligonucleotides A and B, are generally comprised of DNA. However, also included are probes made up of or including ribonucleotides or synthetic or modified nucleotide residues that are capable of participating in Watson-Crick type or analogous base-pair interactions. Thus, also included are probes comprised of DNA analogues or modified DNA, e.g. PNA or other derivatives including non-nucleotide backbones.

Thus, the partially double-stranded probe used in the method of the invention is comprised of two hybridised oligonucleotides, or two parts of a single, partially self-complementary oligonucleotide, A and B. Oligonucleotide A acts to template the ligation of the target fragment to oligonucleotide B and therefore comprises a first target-specific part and a second non-target-specific part. By "first target-specific part" is meant a part of oligonucleotide A of at least 10 nucleotides in length which is complementary in sequence to at least part of the single-stranded portion of the target fragment. The reference in step (c)(i) to "at one end" is meant in a general sense, i.e. that the first target-specific part and the second non-target-specific part are generally at opposite ends of oligonucleotide A, and does not require that the first target-specific part is at the very terminus of oligonucleotide A. "Complementary" as used here is as defined above, i.e. functionally complementary (capable of mediating

hybridisation). Hence, "complementary" refers to said part as a whole rather than the individual nucleotides and may not necessarily mean 100% complementarity between the first target-specific part of oligonucleotide A and the part of the single-stranded portion of the target fragment to which it hybridises. However, the furthest-apart target fragment-complementary nucleotides of said part must delineate a stretch of at least 10 nucleotides. Thus, the first target-specific part may be 10 nucleotides long or of any length above 10 nucleotides such as, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 100 or any integer between or above these, providing that the length is sufficient to mediate a productive hybridisation, i.e. a hybridisation sufficient to template a ligation between the target fragment and oligonucleotide B. Such a productive hybridisation does not necessarily require that the terminal nucleotides of the relevant end of oligonucleotide A are comprised within the target fragment-complementary stretch of nucleotides (first target-specific part), and therefore oligonucleotide A may contain one or more terminal nucleotides at that end which are not complementary to the target fragment.

The non-target-specific part of oligonucleotide A will typically be of at least 20 nucleotides in length in order to facilitate a stable, productive hybridisation with oligonucleotide B. If the probe is a single, partially self-complementary oligonucleotide, the intramolecular hybridisation may permit a shorter non-target-complementary region of oligonucleotide A, though in such a case the ligase required in the subsequent ligation step will require a double-stranded region of about 6-10 nucleotides. The length of the non-target-specific part of oligonucleotide A will represent the difference between the length of the target-specific part and a maximum length of oligonucleotide A of about 200 nucleotides. Preferably, the first target-specific part and the second non-target-specific part of oligonucleotide A each consist of 20, 25 or 30 or more nucleotides. Oligonucleotide A may in total be, for example, 16 to 200 nucleotides long, more particularly, 18, 20, 30, 40, 50 or 60 to 100, 120, 150 or 200. A representative length range may yet more particularly be 16, 18 or 20 to 40, 50, 60, 70, 80 or 100, or at the higher end, 30, 40 or 50 to 60, 70, 80, 90, 100, 120, 150 or 200.

Oligonucleotide B must be at least of a length sufficient to maintain a productive hybridisation with at least part of the non-target-specific part of oligonucleotide A, but as discussed below may be longer than this and may overhang oligonucleotide A at the other end of the probe from the single-stranded target-specific part. Thus typically, oligonucleotide B will be at least 20 nucleotides long, but may be less, e.g. at least 6, 8 or 10 nucleotides long in the case of a single partially

self-complementary probe. More particularly, a representative oligonucleotide B may thus be 6, 8, or 10 to 20, 30, 40, 50, 70, 80, 100, 120, 150, or 180 nucleotides long.

It will be noted that a stretch of at least 10 nucleotides (representing the first, target-specific, part of oligonucleotide A) is longer than the single-stranded "sticky" overhangs produced by restriction endonucleases. Thus, as used herein "target-specific" indicates a specificity for the target fragment which is based on a stretch of at least 10 nucleotides of the fragment. This means the annealing (hybridisation) of the probe (or more particularly oligonucleotide A of the probe) to the target fragment is dependent on (or contingent on or dictated by) the sequence of the target fragment. The sticky end hybridisation of restriction fragments is not encompassed. The probe is target-specific in the sense of being selective for the target fragment, i.e. capable of hybridising to the target fragment but not to other, non-target, fragments which do not contain a region (or part) of complementarity (in the single stranded portion) to oligonucleotide A of the probe. Thus the probe (or more particularly oligonucleotide A of the probe) discriminates or distinguishes between target and non-target fragments. The person skilled in the art will readily understand that the degree of specificity of the probe for the target fragment may be increased by increasing the length of said first target-specific part. By altering the length of said first target-specific part the "uniqueness" within the nucleic acid sample of the fragment captured by the probe may be varied, and in that way highly similar sequences such as family members or homologues of the target fragment may be included within or excluded from the fragments captured using the probe. Generally, it will be desired that in practice "target-specific" means that said first target-specific part of oligonucleotide A targets a DNA which is unique (albeit possibly present in many copies; a unique species) within the nucleic acid sample. However, as discussed above, it may be desired to use a single probe to detect or enrich for more than one DNA on the basis of shared sequence and in such cases "target-specific" will not mean that the target DNA is unique within the sample. On account of only a single target-specific nucleic acid probe being contacted in step (c) with the fragments of step (b), the method involves only one target-specific probe-binding event per target fragment. More particularly there is only one target-specific probe-binding (probe-hybridisation) event per target fragment before ligation step (d). Put another way, the method does not encompass the use of two or more target-specific probes per target fragment, nor the use of a probe which binds the target fragment more than once in a target-specific manner, in other words at more than one site, and in particular before ligation step (d).

The second non-target-specific part of oligonucleotide A comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe. Hence, the end of oligonucleotide A other than the end containing the first target-specific part hybridises to at least one end of oligonucleotide B. If oligonucleotide B contains a portion non-complementary to oligonucleotide A, this is located at the distal end of oligonucleotide B relative to the first target-specific part of oligonucleotide A. The region of complementarity may be of any length and degree of complementarity capable of mediating a productive hybridisation, i.e. a hybridisation sufficient to template a ligation between oligonucleotide B and the target fragment.

As a result of the templating action of oligonucleotide A which hybridises to oligonucleotide B and the target fragment, oligonucleotide B may be ligated to the target fragment. Oligonucleotide B therefore comprises at least a portion which is complementary in sequence to the second non-target-specific part of oligonucleotide A such that together oligonucleotides A and B form a partially double-stranded nucleic acid probe comprising at one end a single-stranded target-specific part as well as a double-stranded non-target-specific part. In cases where the probe (or more particularly oligonucleotide A) hybridises to the end of the target fragment, the probe may be designed such that, upon hybridisation, oligonucleotide B is positioned immediately adjacent to the end nucleotide of the target fragment. However, as discussed further below this is not an absolute requirement. The end of the probe other than the single-stranded target-specific part may be single- or double-stranded (or a hairpin or hairpin loop, if oligonucleotides A and B are parts of a single oligonucleotide). Thus, reference in step (c)(i) and (ii) above to "at least a portion" of oligonucleotide B means that oligonucleotide B may be longer than the portion of oligonucleotide B complementary to oligonucleotide A, in which case the "overhanging" (protruding) portion non-complementary to oligonucleotide A is at the end of oligonucleotide B other than the end which becomes ligated to the target fragment. Hence, reference in step (c)(i) and (ii) to "including one end" means that the portion of oligonucleotide B which is complementary to the non-target-specific part of oligonucleotide A includes one or, if oligonucleotide B is entirely complementary to (no longer than) the non-target-specific part of oligonucleotide A, both ends of oligonucleotide B. The ligation of oligonucleotide B to a target fragment is the only target-specific probe-binding event that will occur (and more particularly, that will occur up to or before ligation step (d)) for a given target fragment in the method of the invention.

If, as discussed above, oligonucleotide B is longer than the portion of oligonucleotide B complementary to oligonucleotide A, and the overhanging portion is at the 3' end of oligonucleotide B, said 3' end could serve as a primer for rolling circle amplification of a circularised or circularisable nucleic acid molecule hybridised to said 3' end. The nucleic acid may be pre-hybridised to oligonucleotide B or may be added separately, and may be pre-circularised or circularised by ligation as a result of a ligation reaction mediated by hybridisation to oligonucleotide B. Upon addition of DNA polymerisation reagents, an RCA product will be generated from the circularised template, said product being continuous with the probe-target fragment hybrid. Preferably, the polymerase of said polymerisation reagents is phi29 polymerase. Hence, in an *in situ* embodiment where the target nucleic acid fragment is immobilised, the generation of an RCA product may serve to detect the target nucleic acid. The RCA product could be visualised *in situ* by hybridisation of fluorescently-labelled oligonucleotides, for example.

Oligonucleotide B may contain or carry an element by which the target fragment may be detected or enriched. By "contains or carries" is meant that such an element may be contained within the nucleotide sequence of the oligonucleotide, e.g. a probe or primer binding site or other nucleic acid-based affinity-binding site (for example a binding site for a hybridisation probe or for a DNA binding protein etc., which binding site may be viewed as a capture or detection element depending on the nature of the probe or affinity binding element, or a binding site for a sequencing primer, which sequencing primer binding site may be accordingly be viewed as a detection element, or for an amplification primer, which amplification primer binding site may accordingly be viewed as an amplification element), or it may be attached or conjugated or in any way linked or coupled to or associated with oligonucleotide B. For example, it may be a functional moiety (e.g. a chemical group or a molecule) which is attached etc. to the oligonucleotide, such as an immobilisation moiety or a detection moiety (e.g. a reporter or a label). An immobilisation moiety may for example be an affinity binding moiety or group, e.g. one member of an affinity binding pair (i.e. an affinity ligand), which is attached or conjugated etc. to said oligonucleotide, and is capable of binding to the other member of the affinity binding pair (i.e. its cognate binding partner) for the purposes of capture or separation, e.g. when the cognate binding partner is attached to a solid phase.

Oligonucleotide B may contain one or a plurality of such detection or enrichment (e.g. amplification and/or capture) elements.

A detection element may as noted above be a binding site contained in the oligonucleotide sequence (e.g. a binding site for a detection probe or moiety or for a

primer to be used in a detection reaction, e.g. a sequencing primer) or it may be a detection moiety which is carried in any way by the oligonucleotide, e.g. a reporter group or moiety or a label, which may be directly or indirectly signal-giving. For example it may be a visualisable label, such as a coloured or fluorescent or particulate label, or a moiety which contributes to or takes part in a signal-giving reaction, e.g. an affinity binding partner or ligand or a substrate or co-factor for an enzyme.

An enrichment element may be any element for the amplification and/or capture of the target fragment, or indeed for enrichment of the target fragment by any means.

As can be seen from the discussion above an "amplification element" may be any feature of, or attached to, oligonucleotide B which may be used to amplify the target fragment of the probe-target fragment hybrid. Typically it will be an amplification primer binding site. Such an amplification primer binding site may be a binding site for a primer for single-sided amplification or polymerisation runoff, e.g. using T7 RNA polymerase primer to repeatedly prime transcription resulting in amplification (Van Gelder RN et al, Proc Natl Acad Sci U S A. 1990 Mar;87(5):1663-7) or a rolling circle amplification primer (see further below). It may also be a binding site for one of a number or set (e.g. pair) of amplification primers, for example to allow exponential amplification, e.g. a PCR primer or a primer for a PCR-based procedure. As described further below, if more than one amplification primer is used one or more further primer binding sites may be provided to the target fragment in a separate step. The primer binding site may also be used for the binding of a sequencing primer, or a sequencing primer binding site may be located elsewhere in oligonucleotide B.

A "capture element" is any moiety carried by (e.g. attached or conjugated to etc.) oligonucleotide B, or any feature of the sequence of oligonucleotide B (e.g. a binding site), which may potentially be used selectively to attach a probe-bound target fragment (probe-target fragment hybrid) to a solid phase or support, including for example a particle such as a bead. Hence, a capture element may be viewed as an "immobilisation element". Numerous examples of such elements are known in the art and include, e.g., an affinity binding partner, e.g. biotin or a hapten, capable of binding to its binding partner, i.e. a cognate binding partner, e.g. streptavidin or avidin, or an antibody, provided on the solid phase or support. Said interaction between oligonucleotide B and a solid phase may particularly be mediated by click chemistry (Kolb HC et al, Angew Chem Int Ed Engl. 2001 Jun 1;40(11):2004-2021).

The solid phase may be any of the well known supports or matrices which are currently widely used or proposed for immobilisation, separation etc. These may

take the form of particles (e.g. beads which may be magnetic or non-magnetic), sheets, gels, filters, membranes, fibres, capillaries, or microtitre strips, tubes, plates or wells etc. The support may be made of glass, silica, latex or a polymeric material. Suitable are materials presenting a high surface area for binding of the analyte. Such supports may have an irregular surface and may be e.g. porous or particulate e.g. particles, fibres, webs, sinters or sieves. Particulate materials e.g. beads are useful due to their greater binding capacity, particularly polymeric beads. Conveniently, a particulate solid support used according to the invention will comprise spherical beads. The size of the beads is not critical, but they may e.g. be of the order of diameter of at least 1 and preferably at least 2 μm , and have a maximum diameter of preferably not more than 10, and e.g. not more than 6 μm . Monodisperse particles, that is those which are substantially uniform in size (e.g. size having a diameter standard deviation of less than 5%) have the advantage that they provide very uniform reproducibility of reaction. Representative monodisperse polymer particles may be produced by the technique described in US-A-4336173. However, to aid manipulation and separation, magnetic beads are advantageous. The term "magnetic" as used herein means that the support is capable of having a magnetic moment imparted to it when placed in a magnetic field, and thus is displaceable under the action of that field. In other words, a support comprising magnetic particles may readily be removed by magnetic aggregation, which provides a quick, simple and efficient way of separating the particles following the analyte binding steps. Particularly advantageous solid phases include very small particles which can efficiently contact a high proportion of the immobilisable oligonucleotide Bs. Such particles may further be useful by retarding the movement of particle-attached target fragments through a gel, allowing separation from free, non-particle-attached (non-target) fragments. Alternatively, also preferred is the use of a chromatographic matrix modified with groups that can be reacted covalently or non-covalently with groups on oligonucleotide B of the probe.

As noted above, enrichment and/or detection of the probe-target fragment hybrid can occur by any means selective for the hybrid. As described further below, in certain embodiments of the invention the probe-target fragment hybrid may be circularised. In such an embodiment enrichment for the hybrid may take place by enriching for circular molecules, e.g. by degrading any non-circular (i.e. linear) nucleic acid molecules using an exonuclease.

Oligonucleotide B may further contain a recognition sequence for a restriction endonuclease such that the partially double-stranded, intact probe formed from the hybridisation of oligonucleotides A and B is endonucleolytically cleavable. In

particular, the recognition sequence may be for a rare, infrequently-cutting endonuclease. Such a cleavage site may be useful in releasing immobilised probe-target fragment hybrids from the solid phase.

Alternatively or additionally, oligonucleotide B may contain a "molecular tag", i.e. a feature which allows an oligonucleotide B used in the method of the invention as performed on a particular sample to be distinguished from an oligonucleotide B used in the method as performed on a different sample and which thereby allows identification of the sample from which a given fragment has been enriched or detected. The molecular tag may be contained in a portion of oligonucleotide B non-complementary to oligonucleotide A. The samples may, for example, correspond to patient samples. In such an embodiment a different oligonucleotide A would be needed for each target nucleic acid, whilst the oligonucleotides B would need to differ only between the method as performed on different samples.

As discussed above, the method of the invention may be performed in multiplex. In such an embodiment, the respective oligonucleotide Bs of the plurality of nucleic acid probes comprise a common sequence which is the same in each probe. Thus, in the context of oligonucleotide B of the probe, "common" refers to a sequence of oligonucleotide B, which may comprise all of oligonucleotide B, which is the same (generic) amongst the plurality of probes used in a multiplex reaction. In particular, the common sequence in each probe may comprise the detection and/or enrichment element (e.g. amplification and/or capture element), whereby the plurality of different target fragments may be amplified and/or captured together, and/or the restriction endonuclease recognition sequence. More particularly, the plurality of nucleic acid probes in a multiplex aspect of the method comprise the same oligonucleotide B.

Once the target fragment is hybridised at one end, and oligonucleotide B is hybridised at least at one end, to oligonucleotide A of the probe, the respective ends of the target fragment and oligonucleotide B may be ligated to produce a probe-target fragment hybrid. As mentioned above, and discussed further below, a target fragment "end" for ligation may be created by hybridising a probe (or oligonucleotide A of a probe) internally in the target fragment in such a manner that a substrate for a flap endonuclease is formed, and cleaving such a structure with a flap endonuclease. The probe-target fragment hybrid comprises the target fragment ligated to the probe.

Enzymes appropriate for the ligation step are known in the art and include, e.g. Tth DNA ligase, Taq DNA ligase, *Thermococcus* sp. (strain 9^oN) DNA ligase (9^oNTM DNA ligase, New England Biolabs), AmpligaseTM (Epicentre Biotechnologies) and T4 DNA ligase. The ligation of oligonucleotide B and the target fragment may

occur directly, i.e. the respective ends are immediately juxtaposed on hybridisation to oligonucleotide A, or indirectly, in which case the hybridised ends are separated by a gap. In the latter case, the gap may be filled by an added oligonucleotide complementary to the region of oligonucleotide A in the gap (i.e. a "gap" oligonucleotide), necessitating two ligation reactions, or alternatively the gap may be filled by polymerase extension of one of the ends until the extended end meets the other end, the immediately juxtaposed ends then being ligated together. Polymerases suitable to perform such a "gap-filling" reaction are known in the art.

In aspects of the method in which the fragments of the nucleic acid sample are rendered partially (incompletely) single-stranded, after the step of ligating the probe to the target fragment a polymerase optionally may be added to the reaction to repair (fill-in) any gap between the target fragment-hybridised end of oligonucleotide A of the probe and the exonucleolytically-degraded end of the corresponding strand of the target fragment. The polymerase may extend said end of oligonucleotide A or said end of the corresponding strand of the target fragment, whichever of these ends is a 3' end. The polymerase may be a strand-displacing or a non-strand-displacing polymerase. In the former case the polymerase will, after filling said gap, continue to the end of the template by displacing (and "replacing") oligonucleotide A or the corresponding strand of the target fragment as the case may be (i.e. depending on whether the polymerase is extending from the end of oligonucleotide A or from the end of the corresponding strand of the target fragment). Such strand-displacing polymerases are known in the art and include, e.g., phi29 DNA polymerase. Non-strand-displacing polymerases may also be used and are known to include, e.g., T7 DNA polymerase. Such a polymerase will stop once it has repaired the gap and encounters double-stranded nucleic acid. The extended end may then be ligated to the end of, as the case may be, oligonucleotide A of the probe or the corresponding strand of the target fragment. In this way the double-strandedness of the probe-target fragment hybrid is substantially restored. After the ligation, and optional gap-filling, steps, unbound probe may optionally be removed from the reaction by a size separation step.

As discussed above, the probe-target fragment hybrid may be detected or enriched for by any means selective for the hybrid, for example by degradation of nucleic acid other than the hybrid or by means of a detection and/or enrichment element in oligonucleotide B. As also discussed above, such an enrichment element may be a capture, or immobilisation, element, which may be any moiety attached to, or stretch of the nucleotides of, oligonucleotide B capable of interacting with an element on a solid phase or support.

By means of the capture element the probe-target fragment hybrid may be separated (enriched) from the non-target fragments for, by example, washing or gel purification. As discussed above, the covalent nature of the ligation between the probe and the target fragment permits the use of highly stringent washes effective to remove fragments which are not ligated to the probe. Such washes are not possible in known methods in which the probe and target fragment are annealed through hybridisation, and therefore the ability to use such high stringency conditions in the present method is advantageous. Alternatively, if particles are used, another option is size selection such as gel purification.

The method of the invention may be performed with a probe having an oligonucleotide B which is immobilisable, i.e. carrying or containing an immobilisation (capture) element, or which is immobilised, i.e. wherein said immobilisation element-carrying oligonucleotide B is already bound to a solid phase. In such a latter embodiment, the step of enriching constitutes washing the immobilised probe-target fragment hybrid to remove non-target fragments.

A target fragment captured, or immobilised, as described above may directly be subjected to analytical techniques such as sequencing, or may instead firstly be amplified. Alternatively, the target fragment may not be captured/immobilised but may instead simply be amplified and/or sequenced. Thus, amplification of the target fragment may be by any suitable method for amplifying nucleic acids from a single amplification primer binding site (i.e. in oligonucleotide B), such as by the use of a T7 RNA polymerase primer, as discussed above. Sequencing reactions may be primed from such an amplification primer binding site, if present, or a separate sequencing primer binding site within oligonucleotide B. As used herein, reference to sequencing the target fragment means sequencing (i.e. determining the identity thereof) at least two consecutive nucleotides in the target fragment. As described further below, amplification may also be performed by methods which involve more than one amplification primer. A binding site for such a second or further primer may be provided to the target fragment in a separate step, as described below. Such a further amplification primer binding site will not be target fragment-specific, or will not be provided to the target fragment in a target-specific manner.

It may be desirable to release the immobilised, enriched fragments from the solid phase to facilitate their analysis. This may be achieved by cleaving in the probe of the probe-target fragment hybrid by, e.g., a restriction endonuclease having a recognition site in oligonucleotide B of the probe, as discussed above. Preferably, the restriction endonuclease is a rare-cutting endonuclease. If the immobilised probe-target fragment hybrid has become single-stranded, it may be necessary to add an

oligonucleotide to render the appropriate part of the probe double-stranded prior to cleaving. Released target fragments may then be subject to analytical techniques, such as those discussed above. Such techniques may be performed directly on the released fragments, or on amplification products thereof. In the latter case, the released fragments may be inter- or intramolecularly ligated to give linear concatemers or double-stranded circular molecules. This may require modification of the ends of the released fragments, e.g. by restriction digestion and optionally enzymatic blunting using e.g. Klenow fragment of DNA polymerase I that fills in 5' overhangs by polymerase activity and degrades 3' overhangs with exonucleolytic activity, thereby converting sticky ends to blunt ends. Circular molecules may be amplified by rolling circle amplification (RCA). For sequencing, sequences within oligonucleotide B of the probe, may be used to bind sequencing primers.

Numerous variations of the general method of the invention are contemplated, several of which are described below.

After step (a) above but before step (b) an optional further step may be performed in which a common nucleic acid adaptor is non-target-specifically annealed to the ends of the fragments. In this context, "common" means that the same adaptor is annealed to all fragments (or to a number of fragments - see further below - but in any case to more fragments than solely the target fragment(s)) within a given sample and is therefore common, or generic, to the fragments, or to a sub-population of fragments, of that sample, in contrast to the probe which anneals only to the target fragment. In cases where more than one enzyme, e.g. restriction endonucleases, have been used to fragment the sample, more than one "species" of adaptor may be used, wherein such adaptors are identical except for one "annealing end" in each case which differs to "match" the differing fragment ends produced by the enzymes.

The nucleic acid adaptors of the invention are substantially double-stranded molecules. However, by "double-stranded" in the present context does not mean (but does encompass) that said ends are "blunt", and "sticky" ends consisting of a single-stranded overhang of 1, 2, 3, 4, 5 or 6 etc. nucleotides generated by restriction enzyme digestion are included in this meaning. The adaptors may be of any suitable length.

By "non-target-specifically" is meant that said adaptor is annealed by a means which has no specificity for the target fragment in the sense of "target-specific" as defined elsewhere herein. In other words the adaptor is not selective for the target, or for non-target fragments, i.e. it does not discriminate or distinguish between target or non-target fragments. Thus, the binding (hybridisation) of the

adaptor is not dependent or contingent upon a nucleotide sequence which is particular to (or specific for) the target fragment relative to the non-target fragments. As a result, such an adaptor is annealed to the ends of all of the fragments (including the target fragment). This is unlike the annealing of the probe of step (c) which is targeted to the target fragment by virtue of the complementary sequence of prescribed length present in oligonucleotide A of the probe. Such non-target specific annealing may be achieved by any method which facilitates the annealing of the adaptor to all of the fragments, such as through annealing with sticky restriction ends (if present) of the fragments, or blunt- or artificially-created sticky-end annealing. Hence, the adaptors must be designed with at least one end suitable for such annealing. For example, sticky ends of the fragments may be blunted by the use of a suitable polymerase, and single adenosines added as 3' overhangs, again through the action of an appropriate polymerase. Examples of suitable polymerases for either purpose are known in the art and include, in the former case, T4 DNA polymerase, and in the latter case Taq polymerase. If the adaptor is designed with a 5' thymidine overhang, the adenosine and thymidine overhangs will act as complementary sticky ends susceptible to ligation (known as "TA ligation"), promoting ligation of adaptors to fragments. The adaptors may be modified at one end to reduce or prevent ligation of adaptors with each other by any means which prevent ligation but do not otherwise interfere with the assay. Such modifications include the absence of 5' phosphate or 3'OH groups, addition of fluorophores or affinity groups, and the introduction of "blocking" groups such as amine groups.

Importantly, whilst said adaptor may become annealed to both ends of the fragments, it becomes ligated only at the 3' ends, or only at the 5' ends, of the strands of the fragments (thus, "annealed" is meant as defined above, i.e. it does not include ligation). This is in order that it can be ensured that, for the target fragment, an adaptor is ligated to the same strand to which oligonucleotide B will become ligated in step (d), but at the other end of that strand. Selective ligation of the adaptor to only the 3' ends, or only the 5' ends, of the strands of the fragments may be achieved by any suitable means. For example, ligation of the annealed adaptor to either the 3' or 5' ends of the strands may be controlled by dephosphorylation of the fragments or the adaptors, respectively. Suitable dephosphorylases (phosphatases) are known in the art and include, e.g., Antarctic phosphatase (New England Biolabs), shrimp alkaline phosphatase and calf intestinal phosphatase. Ligation of the fragments and adaptors may be achieved using suitable ligases as known in the art, such as T4 DNA ligase. The addition of single adenosines as 3' overhangs to the

blunted fragments, and/or dephosphorylating said fragments, is also beneficial in reducing or avoiding inter-fragment ligation.

In one embodiment, therefore, the method of the invention further comprises, between steps (a) and (b), the step of non-target-specifically annealing to the ends of said fragments a common (or generic) nucleic acid adaptor, wherein the annealed adaptor becomes ligated to the fragments only at the 3' ends, or only at the 5' ends, of the strands of the fragments. As mentioned above, a single species of adaptor may be used, or one or more adaptors may be used, depending on the fragmentation procedure. Thus, the adaptor is annealed such that an adaptor is ligated to one end of the strand of the target fragment to which, at the other end, oligonucleotide B of the probe is ligated in step (d). In other words, in the target fragment the adaptor is ligated at the opposite end of the strand to which the target-specific probe becomes ligated.

In a further embodiment, the ends of the fragments are rendered sticky with respect to at least one end of the adaptor prior to ligation. In a still further aspect, dephosphorylation of the fragments or the adaptor is used to facilitate ligation of the adaptor to, respectively, either the 3' ends or the 5' ends of the strands of the fragments.

In embodiments of the method of the invention in which the adaptor-ligated target fragments are rendered completely single-stranded in step (b), the method must be designed so that the specificity of the subsequently-contacted probe is for a part of the target fragment which results in ligation of oligonucleotide B of the probe to the other end of the target fragment relative to the end to which the adaptor is ligated. Hence in such an aspect, the first target-specific part of oligonucleotide A comprises at least 10 nucleotides complementary in sequence to a portion at the end of said single-stranded target fragment other than the end to which the common nucleic acid adaptor is ligated.

The adaptor, which if used in the method of the invention will become comprised in the probe-target fragment hybrid, may contain an element (advantageously a sequence) useful for the enrichment and/or detection of the target nucleic acid. In particular, such an element or sequence will be used in conjunction with an element present in the probe. It will thus be appreciated that such an element may be a binding site for one of an amplification primer pair (or number or set of primers), the binding site(s) for the other(s) of which are provided in the probe. Accordingly, in one embodiment, the common nucleic acid adaptor comprises an element for amplification, preferably an amplification primer binding site. For example, the adaptor may comprise an amplification primer binding site orientated

appropriately with respect to another such binding site in the probe such that an amplification product may be generated when the target fragment is ligated to both the probe and the adaptor. Such amplification may be by, e.g., the polymerase chain reaction (PCR), of which many modified versions (e.g. "real-time" or quantitative PCR) are well known in the art, or other amplification methods. The amplification product may be analysed by, e.g., massive parallel sequencing platforms (e.g. SOLiD (Applied Biosystems, Inc.), Illumina Genome Analyzer (Illumina, Inc.), Genome Sequencer (454 Life Sciences)) microarrays or hybridisation-based sequencing.

Said amplification primer binding site, or a separate sequence located elsewhere in the adaptor, may be used as a binding site for a sequencing primer for sequencing the enriched target nucleic acid.

Additionally or alternatively to the presence of an amplification element, the common nucleic acid adaptor may carry a molecular tag. By "molecular tag" is meant a feature which can be used to differentiate between otherwise identical adaptors. Put another way, such a tag is an element which may be used to generate distinguishable variants of a single adaptor. The tag may be a particular stretch of the adaptor's nucleotide sequence.

As discussed above, the nucleic acid sample on which the method of the invention is performed may be comprised of a plurality of pooled nucleic acid samples of different origins. Such pooling to give a single nucleic acid sample may occur after fragmenting step (a). In such an aspect, adaptors carrying different molecular tags may be ligated to the fragments of the respective nucleic acid samples prior to pooling, such that in the pooled nucleic acid sample subjected to the remaining steps of the method the fragments originating from different samples will be ligated to adaptors distinguishable by virtue of the different molecular tags. This is highly advantageous as it allows the simultaneous (parallel) analysis, in a simplex (one probe, for one or a plurality of sufficiently similar target nucleic acids) or multiplex (a plurality of probes, for a plurality of target nucleic acids) format, of multiple samples. For example, one or more target nucleic acids may be detected or enriched for in a single assay, from a plurality of differently-tagged patient samples. The use of molecularly-tagged common nucleic acid adaptors to identify the fragments of the respective samples, instead of incorporating tags in the target-specific probes, vastly reduces the number of different probes which would need to be synthesised in order to process multiple samples (containing a plurality of target nucleic acids) in parallel.

In one embodiment, therefore, in step (a) said fragmenting a nucleic acid sample comprises separately fragmenting a plurality of nucleic acid samples, ligating

to the fragments of which samples, respectively, common (or generic) nucleic acid adaptors carrying different molecular tags, and pooling said adaptor-ligated fragmented nucleic acid samples prior to step (b).

The adaptor may further comprise a recognition site for an endonuclease.

In another aspect of the method of the invention, the hybridisation and ligation steps (c) and (d) occur through resolution of a generated secondary structure using a flap endonuclease (FEN). In order to generate the secondary structure which is the substrate for the flap endonuclease, the first target-specific part of oligonucleotide A of the probe must be complementary to a portion internal to the target fragment, rather than to an end portion. Hybridisation of the probe (comprising oligonucleotide A hybridised to oligonucleotide B) to the target fragment causes the non-hybridised 5' end of the target fragment to protrude. The resulting secondary structure is recognised by a flap endonuclease which cleaves off the protruding non-hybridised 5' end of the target fragment to reveal a new 5' end which is hybridised to oligonucleotide A and is ligatable to the oligonucleotide B. Thus, the part of the single-stranded portion of the target fragment which is ligated to oligonucleotide B of the probe (i.e. the part that is hybridised to oligonucleotide A of the probe as described in step (d)) is said ligatable 5' end, and the probe-target fragment hybrid will lack a portion at the 5' end of the original, pre-probe-bound target fragment corresponding to said protruding end.

In such an embodiment, therefore, the first target-specific part of oligonucleotide A comprises at least 10 nucleotides complementary in sequence to an internal non-end portion of said single-stranded target fragment, and said annealing of said probe to said target fragment is through hybridisation of said internal non-end portion to the first target-specific part of oligonucleotide A causing the 5' end of the target fragment to form a substrate for flap endonucleolytic cleavage and further comprising cleaving said flap endonucleolytic cleavage substrate to produce a ligatable 5' end of said target fragment which is hybridised to oligonucleotide A of said probe.

As such a use of a flap endonuclease results in internal cleavage of the target fragment to generate a 5' end for ligation to oligonucleotide B of the probe, if a common nucleic acid adaptor is used said adaptor must not be ligated only to the 5' ends of the strands of the fragments. Thus, the adaptor may be ligated to both the 5' and 3' ends of the strands of the fragments (in which case the 5' end adaptor will be cleaved off during the flap cleavage step) or may be ligated to only the 3' ends of the strands of the fragments (which can be achieved by, for example, dephosphorylation of the fragments as described above).

Hence in a further embodiment, if a common (or generic) nucleic acid adaptor is non-target-specifically annealed to the ends of the fragments between steps (a) and (b), said annealed adaptor becomes ligated to the fragments only at the 3' ends, or at both the 3' and 5' ends, of the strands of the fragments, more particularly such that an adaptor is ligated to at least the 3' end of the strand of the target fragment to which, at the 5' end, oligonucleotide B of the probe is ligated in step (d).

In another aspect of the method of the invention, the probe-target fragment hybrid is circularised. In such a case enriching may involve increasing the amount of circular molecules relative to the amount of linear molecules. Thus, following the ligation step (d), an end of at least the target fragment, being the end to which the probe is not ligated (which end may in certain embodiments comprise a common nucleic acid adaptor) is rendered double-stranded in the event that said end is not double-stranded following the step (b) of rendering the fragments at least partially single-stranded (e.g. in aspects of the method wherein the fragments were made completely single-stranded). "Double-stranded" in the present context does not mean (but does encompass) that said ends are made "blunt", and "sticky" ends formed by a single-stranded overhang of 1, 2, 3, 4, 5 or 6 etc. nucleotides generated by restriction enzyme digestion are included in this meaning. Such rendering double-stranded may be achieved by any suitable method, e.g. by polymerisation from a hybridised probe 3' end or from added oligonucleotides (e.g. hexamers) annealed to the target fragment or, if present, adaptor. In embodiments in which an adaptor is ligated to the fragments, the end (comprising said adaptor) may be made double-stranded by adding an oligonucleotide complementary to the adaptor. In embodiments wherein the fragments are rendered only partially single-stranded, the ends will typically already be double-stranded and no such action is required.

Said double-stranded end of the target fragment is then non-target-specifically annealed intramolecularly with the free, non-target bound end of the probe at the other end of the probe-target fragment hybrid. As the remaining, non-target, fragments will lack ligated probe and therefore be single-stranded at the other end, such intramolecular annealing will not occur. Thus, the probe-target fragment hybrid, optionally also containing a common nucleic acid adaptor, will intramolecularly adopt a circular conformation. In this context, by "non-target-specifically" is meant that said annealing is by means which do not take advantage of or rely on (i.e. are not dependent upon) the particular nucleotide sequence of the target fragment in the sense that no knowledge of the sequence of the target fragment is required in order to effect such annealing. Hence, the means of

annealing may differ according to whether or not the relevant end of the target fragment comprises an adaptor. If an adaptor is present, such annealing will include that between sticky restriction ends, as no sequence of the target fragment is required to exploit a restriction recognition sequence within the known sequence of the adaptor. However, if no adaptor is present, such annealing is limited to that between two blunt ends or two "artificial" sticky ends (such as used in TA-ligation, discussed above). In such embodiments the probe is designed to have, at the free, non-target fragment-bound end, a blunt or complementary sticky end, as appropriate, or contains a restriction endonuclease recognition sequence allowing the creation, through cleavage, of such an end. After the intramolecular-annealing of the probe-target fragment hybrid, oligonucleotide B of the probe is ligated with the corresponding strand of the double-stranded end resulting in circularisation of the hybrid.

Hence, in a further embodiment the method further comprises, between steps (d) and (e), rendering double-stranded an end of at least the target fragment, being the end to which the probe is not ligated, which end will comprise a common nucleic acid adaptor sequence in the case that such an adaptor is ligated to the fragments between steps (a) and (b); non-target-specifically annealing said double-stranded end intramolecularly with the free, non-target fragment-bound end of the probe; and ligating oligonucleotide B of the probe-target fragment hybrid with the corresponding strand of said double-stranded end to circularise said hybrid.

The selective circularisation of the target fragment in this aspect of the method can be exploited in the enriching and/or detecting step. In one embodiment, therefore, said enriching and/or detecting is by means that increases the ratio of circular to linear nucleic acids. Such enrichment or detection of circular molecules may be achieved by any suitable means, which include methods to selectively amplify circular nucleic acids, e.g., rolling circle replication (RCA), hyperbranched RCA, multiple strand displacement. Furthermore, enrichment of circular molecules may be achieved by methods to selectively remove non-circular nucleic acids, e.g. exonucleolysis. Suitable exonucleases are known, and include exonuclease I, exonuclease III, lambda exonuclease.

A number of representative preferred specific embodiments of the invention are enumerated below.

In a first such embodiment, the method of the invention comprises:

(a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target nucleic acid;

- (b) non-target-specifically annealing to the ends of said fragments a common nucleic acid adaptor, wherein the annealed adaptor becomes ligated to the fragments only at the 3' ends, or only at the 5' ends, of the strands of the fragments;
- (c) rendering said fragments, including said target fragment, single-stranded;
- (d) contacting the single-stranded fragments of step (c) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:
 - (i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to a portion at the end of said single-stranded target fragment other than the end to which the common nucleic acid adaptor is ligated in step (b), and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and
 - (ii) oligonucleotide B is a single-stranded oligonucleotide which contains or carries an amplification primer binding site and optionally an element for immobilisation to a solid phase, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A,such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment ;
- (e) ligating oligonucleotide B of said probe to the end of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid; and
- (f) enriching for said probe-target fragment hybrid through amplification by means of the amplification primer binding site in oligonucleotide B of the probe and an amplification primer binding site in the common nucleic acid adaptor, and optionally immobilising said fragment to a solid phase by means of the immobilisation element in oligonucleotide B of the probe.

Preferably, the fragmenting in step (a) is by restriction endonuclease and/or in step (c) the fragments are rendered single-stranded by denaturation.

As discussed above, the method of the invention is advantageous in that the target fragment undergoes only a single target-specific binding event with the probe, necessitating knowledge of only a single, short region of the target fragment. This is an advantage over known methods such as PCT and the Selectors of WO 2005/111236 which require knowledge of the target fragment/nucleic acid sequence

at two regions (which delineate the target nucleic acid). In particular, the method of the invention facilitates the analysis of unknown nucleic acid junctions such as DNA translocation breakpoints, recombination sites and splice junctions. The single-sided ligation of the probe with the target nucleic acid fragment, containing the target nucleic acid, can be thought of as the fragments being selectively "pulled out" of the nucleic acid sample by one end (the end containing known sequence, on the basis of which the probe is designed). Providing the sample has been fragmented appropriately, i.e. to give fragments of sufficient length, the junction of interest will be located in the pulled-out fragment and may be analysed and identified by, for example, nucleic acid sequencing. The method clearly has potential application in the field of medical diagnostics, particularly given the ease with which the method may be multiplexed through the use of target-specific probes having the same enrichment elements in oligonucleotide B, and optionally the use of a common adaptor. Whilst such an adaptor, where used, will be common in terms of all "functional" elements (such as amplification elements or restriction endonuclease recognition sites), as described above distinguishable variants may be generated by adding molecular tags. Such differently-tagged adaptors may respectively be ligated to different fragmented sub-samples of the single (pooled) nucleic acid sample which is subjected to the method. In addition to allowing the rapid preparation of hundreds or thousands of samples in a single probing and enrichment step, this facilitates ease of identification of target nucleic acids enriched from such samples in parallel, which in a medical diagnostic context may be patient samples. The use of such tagged adaptors is advantageous in dramatically reducing the number of individual probes which would need to be designed and synthesised in order for the sample provenance of each targeted nucleic acid to be indicated by use of "tagged" probes. Furthermore, the probe, and if used the adaptor, may be designed to comprise the primers used for sequencing, thereby forming an amplification product that can be sequenced directly with very little additional preparation.

The single-sided ligation feature of the method further enables other useful applications. For example, the fact that a target-specific probe-binding event occurs only at one "end" of the target fragment, with the other end binding non-target-specifically to a common adaptor (where used), means that the method may have use in analysis of degraded DNA which may be present in scarce amounts, such as in histological samples (e.g. formalin-fixed, paraffin-embedded samples). Since the adaptors are non-target-specifically ligated to the target fragments, every probe-bound fragment will also have an adaptor ligated thereto even if the non-probe-bound end has been generated by degradation or e.g. physical, "random" fragmentation. As

a result, the method may facilitate enrichment of nucleic acids from samples not amenable to enrichment by other methods which require target-specific binding at both ends of the target nucleic acid (e.g. PCR or methods relying on intramolecular circularisation). Further, such known methods may require the target fragment to be of a minimum length, for efficient amplification, whereas fragment length is not critical in the method of the invention. Hence, the method could be used on degraded or fragmented nucleic acid with very short average fragments, representing a suitable approach for analysis of degraded DNA in scarce amounts.

As discussed above, a further advantage of the method of the invention is the covalent nature of the connection (ligation) between the target fragment and the probe. The probe becomes directly ligated to the target fragment of the sample (not to an amplification or polymerisation product thereof), allowing the use of stringent separation conditions to effectively remove free probe and non-target fragments and other reagents leading to enrichment relative to prior art methods such as microarray capture and FISH which rely on hybridisation between the probe and the target.

The method, which in most embodiments does not rely on circularisation of the target fragment, is for that reason particularly suitable for enriching for long genomic target fragments, such as fragments over 2kb in length which can be more difficult to circularise due to the lower proximity of fragment ends relative to shorter fragments. This makes the method very suitable for analysing, by e.g. sequencing and/or high resolution polymorphism analysis, genomic regions identified by whole genome association studies to be associated with disease states. The enrichment of long fragments is also useful in another application enabled by the method of the invention, namely haplotype analysis. If the sample is known to contain a heterozygous polymorphism, it may be possible to fragment the sample using one or more enzymes including one which recognises a site inactivated by the polymorphism. By designing probes respectively to target fragments generated by the presence and absence of cleavage at the polymorphic sites, fragments corresponding to each haplotype may separately be enriched allowing analysis/identification of the respective haplotypes. In such an aspect, the generation and enrichment of long fragments is desirable to maximise the amount of haplotype-specific nucleic acid available for analysis.

As the method generally represents a highly specific way to detect and amplify particular nucleic acids from a complex sample, it may be used in preference to known methods for analysing microbes and other exogenic pathogens in complex DNA samples. The probes of the invention are shorter and cheaper to make than padlock probes, known in the art. The foreign nucleic acid could be amplified and

analysed by, e.g., sequencing, in order to characterize the infectious agent for, e.g., diagnostic or drug sensitivity screening purposes.

Unlike, e.g. PCR, the method of the invention is compatible with combinatorial oligonucleotide synthesis, e.g. on microarrays, enabling the production of large probe sets at low cost, and allows analysis single molecules.

In a second embodiment of the method of the invention, said method comprises:

- (a) fragmenting a nucleic acid to generate nucleic acid fragments including a target fragment containing said target nucleic acid;
- (b) rendering said fragments, including said target fragment, partially single-stranded by 3' or 5' exonuclease digestion, wherein the length of the resulting single-stranded end portion is sufficient to allow hybridisation of at least part of the single-stranded end portion of said target fragment to the probe of step (c);
- (c) contacting the partially single-stranded fragments of step (b) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:
 - (i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part of said single-stranded portion (or more particularly to a portion at the end of said single-stranded portion) of said target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and
 - (ii) oligonucleotide B is a single-stranded oligonucleotide which contains or carries an element for immobilisation to a solid phase and optionally an amplification primer binding site, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A,such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment ;
- (d) ligating oligonucleotide B of said probe to the end of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid; and
- (e) enriching for said probe-target fragment hybrid through immobilisation to a solid phase by means of the immobilisation element in oligonucleotide B of the probe, and optionally by amplifying said target fragment by means of the amplification primer binding site in oligonucleotide B of the probe.

Such an embodiment is particularly advantageous in that the fragments remain largely or at least partially double-stranded, i.e. are not rendered completely single-stranded. This avoids to some extent the undesirable cross-reactivity between single-stranded nucleic acid fragments and thereby reduces the incidence of hybrids which must be distinguished between. This is especially for the enrichment of long genomic sequences which are more prone to such cross-reactivity when single-stranded. Applications of the method of the invention involving the enrichment of long fragments are discussed above.

Preferably, the fragmenting in step (a) is by restriction endonuclease. In another preferred aspect there exists between steps (d) and (e) a step of contacting the probe-target fragment hybrid with a strand-displacing or a non-strand-displacing polymerase such that any gap between the target fragment-hybridised end of oligonucleotide A of the probe and the exonucleolytically-degraded end of the corresponding strand of the target fragment is filled, substantially restoring the double-strandedness of the probe-target fragment hybrid. In a further preferred aspect, the ligation step is followed by a size separation step to remove free non-target fragment-bound probes.

In a third representative specific embodiment of the method of the invention, said method comprises:

(a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target nucleic acid;

(b) optionally, non-target-specifically annealing to the ends of said fragments a common nucleic acid adaptor, wherein the annealed adaptor becomes ligated to the fragments at the 3' ends or at the 3' and 5' ends of the strands of the fragments such that an adaptor is ligated to at least the 3' end of the strand of the target fragment to which, at the 5' end, oligonucleotide B of the probe is ligated in step (f);

(c) rendering said fragments, including said target fragment, single-stranded;

(d) contacting the single-stranded fragments of step (c) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:

(i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to an internal non-end portion of said single-stranded target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and

(ii) oligonucleotide B is a single-stranded oligonucleotide which contains or carries at least one element for detection and/or enrichment (or more particularly detection, amplification and/or capture) of said target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A, such that said target fragment becomes annealed at said internal non-end portion to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment, wherein said hybridisation causes the 5' end of the target fragment to form a substrate for flap endonucleolytic cleavage;

(e) cleaving said flap endonucleolytic cleavage substrate to produce a ligatable 5' end of said target fragment which is hybridised to oligonucleotide A of said probe;

(f) ligating oligonucleotide B of said probe to the part of the single-stranded portion of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target-fragment hybrid;

(g) enriching for said probe-target fragment hybrid by means of said detection and/or enrichment (or more particularly detection, capture and/or amplification) element.

In this embodiment, the use of a flap endonuclease is advantageous in that it avoids the need to identify an enzyme, e.g. a restriction endonuclease, recognition sequence near to the target nucleic acid in the nucleic acid sample. The sample may be fragmented by any one or more enzymes, even if such enzymes are not known to cut in the proximity of the target nucleic acid. Alternatively, non-sequence-specific fragmentation methods, which produce fragment ends of unpredictable sequence, such as physical methods, e.g. sonication or nebulisation, may be used. In such an embodiment the probe is designed to target a region of known sequence of the target nucleic acid which is internal to a fragment, i.e. is not situated at a fragment end as in other embodiments. Because of this, the sequence at the fragment ends is not important and does not need to be known. The flap endonuclease, recognising the secondary structure (protruding (non-hybridised) 5' region of the target fragment hybridised to the probe), cleaves the fragment to create a "new" 5' end which is hybridised to oligonucleotide A of the probe and is ligatable to oligonucleotide B. The method of the invention is therefore not limited the use of sequence-specific fragmentation of the sample, or to instances wherein the region of known sequence of the target nucleic acid contains an endonuclease recognition sequence.

Preferably, said fragmenting in step (a) is by restriction endonucleolysis and/or in step (c) said fragments are rendered single-stranded by denaturation. Also preferred is that oligonucleotide B contains or carries an amplification primer binding site and optionally an element for immobilisation to a solid phase such that in step (g) said enriching is through amplification by means of the amplification primer binding site in oligonucleotide B of the probe or, if a common nucleic acid adaptor is non-target-specifically annealed to the ends of the fragments between in step (b), said amplification is by means of the amplification primer binding site in oligonucleotide B of the probe and an amplification primer binding site in the common nucleic acid adaptor. In a further preferred aspect, between steps (f) and (g) there exists a step of size-selecting the probe-target fragment hybrid by, e.g., gel purification.

In a fourth representative specific embodiment, the method of the invention comprises:

(a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target nucleic acid;

(b) optionally, non-target-specifically annealing to the ends of said fragments a common nucleic acid adaptor, wherein the annealed adaptor becomes ligated to the fragments only at the 3' ends, or only at the 5' ends, of the strands of the fragments;

(c) rendering said fragments, including said target fragment, at least partially single-stranded, wherein the single-stranded portion includes an end portion and wherein the length of said single-stranded portion is sufficient to allow hybridisation of at least part of the single-stranded portion of said target fragment to the probe of step (d);

(d) contacting the at least partially single-stranded fragments of step (c) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:

(i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part (or more particularly to a portion at the end) of said single-stranded portion of said target fragment, wherein if a common nucleic acid adaptor is ligated to the fragments according to optional step (b) said first target-specific part comprises at least 10 nucleotides complementary in sequence to a portion at the end of said at least partially single-stranded target fragment other than the end to which the common nucleic acid adaptor is ligated; and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and

- (ii) oligonucleotide B is a single-stranded oligonucleotide which contains or carries at least one element for detection and/or enrichment (or more particularly detection, amplification and/or capture) of said target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A, such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment;
- (e) ligating oligonucleotide B of said probe to the part of the single-stranded portion of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target-fragment hybrid;
- (f) if necessary rendering double-stranded an end of at least the target fragment, being the end to which the probe is not ligated, which end will comprise a common nucleic acid adaptor sequence in the case that such an adaptor is ligated to the fragments according to optional step (b);
- (g) non-target-specifically annealing the double-stranded end of step (f) intramolecularly with the free, non-target fragment-bound end of the probe;
- (h) ligating oligonucleotide B of the probe-target fragment hybrid with the corresponding strand of the double-stranded end of step (f) to circularise said hybrid; and
- (i) enriching for said probe-target fragment hybrid by means that increases the ratio of circular to linear molecules.

By circularising the probe-target fragment hybrid, in this embodiment enrichment or detection may advantageously be based on the circularised nature of said hybrids in contrast to the linear form of the non-target fragments.

The step (f) of rendering double-stranded an end of at least the target fragment will not be necessary in embodiments wherein the fragments are rendered only partially single-stranded in part (c) as in such cases the fragment end other than that to which the probe becomes ligated will remain double-stranded. In instances where such rendering double-stranded is necessary, this is preferably achieved by polymerisation from the target fragment-hybridised end of oligonucleotide A of the probe (in cases where this end is a 3' end) or, when a common nucleic acid adaptor is ligated to the fragments, by annealing of an oligonucleotide complementary thereto. The non-target-specific annealing of step (g) may be by blunt or artificially-created sticky end annealing, i.e. by annealing means which does not rely on knowledge of sequence of the target fragment. If a common nucleic acid adaptor is ligated to the target fragment, the annealing may be between sticky ends generated

through sequence-specific digestion of the adaptor and a corresponding sticky end at the free end of the probe.

The enrichment step may involve selective removal of non-circular nucleic acids by e.g. exonuclease treatment. Preferably a mixture of exonucleases enabling degradation of linear double-stranded DNA, for example including exonuclease III, lambda exonuclease and exonuclease I, is used, though any of these or any other suitable exonucleases may be used alone. Alternatively, the circular:linear nucleic acid molecule ratio is increased by selectively amplifying the circularised probe-target fragment hybrid, such as by RCA, hyperbranched RCA or multiple strand displacement.

The invention provides kits for use in the above-described methods. Such a kit will comprise at least an oligonucleotide B and one or more oligonucleotides A, and one or more restriction endonucleases and/or a common nucleic acid adaptor or a set of such adaptors which differ from each other only by molecular tags and/or annealing ends. The oligonucleotide B of the kit may comprise at least one detection and/or enrichment element (more particularly at least one of a detection, capture and/or amplification element) as defined above, and optionally also a cleavage site such as a recognition sequence for an endonuclease. Where one or more restriction endonucleases are included in the kit, such endonucleases will be those which fragment a particular target in a way which renders useful the one or more oligonucleotides A of the kit. If the kit comprises one or more common nucleic acid adaptors, such adaptors will have an annealing end compatible with the sticky ends generated by the one or more restriction endonucleases which fragment a particular target in a way which renders useful the one or more oligonucleotides A of the kit, regardless of whether such one or more restriction endonucleases are included in the kit. The one or more adaptors may contain an amplification and/or sequencing primer binding site, and/or a cleavage site.

The kit may further comprise one or any combination of two or more of the following components. Thus, the kit may contain a ligase for ligating the end of oligonucleotide B of the probe to the target nucleic acid fragment, particularly a ligase as specifically mentioned above in connection with the step of ligating the target fragment to the probe. A solid phase as defined above, to which the oligonucleotide B of the kit is immobilisable, or immobilised, may be included. Alternatively or additionally, an amplification or sequencing primer specific for a primer binding site in oligonucleotide B (i.e. corresponding to sequences of the oligonucleotide B) may be included, and optionally also a polymerase and/or other reagents required for amplification and/or sequencing such as nucleotides, buffers, ions, etc. Means for

cleaving the probe at the cleavage site in oligonucleotide B, such as a restriction endonuclease, may be included. For embodiments of the method of the invention employing an exonuclease for rendering the sample fragments single-stranded, the kit may include such an enzyme, particularly such an enzyme as specifically mentioned above in connection with the rendering single-stranded step. A polymerase may be included for filling any gap between oligonucleotide A and the partially-degraded strand of the probe-hybridised target fragment and/or for extending a 3' end of oligonucleotide A in order to render the non-probe end of the probe-target fragment hybrid double-stranded in certain embodiments. Optional other kit components include a dephosphorylase (phosphatase) such as discussed above, a kinase for adding 5' phosphates for selective ligation, a polymerase (such as T4 DNA polymerase) for blunting overhanging (sticky) restriction fragment ends, free adenosine, a polymerase for adding same to blunted fragment ends, random hexamers for priming polymerisation in order to render the non-probe end of the probe-target fragment hybrid double-stranded in certain embodiments or for multiple strand displacement amplification, qPCR reagents e.g. fluorescently-labelled nucleotides or Taqman® probes, a primer or primers for RCA or hyperbranched RCA, and a flap endonuclease for fragmenting the nucleic acid sample.

The kit may optionally also contain appropriate primers, amplification/sequencing reagents and/or cleaving means as mentioned above for use in connection with an amplification and/or sequencing primer binding site, and/or a cleavage site, in a common nucleic acid adaptor which may be employed in the methods of the invention. Also optionally included in such a kit are single-stranded oligonucleotides corresponding to either strand of the double-stranded adaptor, in order to render the non-probe end of the probe-target fragment hybrid double-stranded in certain embodiments. In certain embodiments hybridisation/ligation of the target fragment to the probe is mediated by a flap endonuclease and accordingly the kit may further include such an enzyme and optionally appropriate co-factors.

The kit components may be present in separate containers, or one or more of the components may be present in the same container, where the containers may be storage containers and/or containers that are employed during the assay for which the kit is designed.

In addition to the above components, the subject kits may further include instructions for practicing the subject methods. These instructions may be present in the subject kits in a variety of forms, one or more of which may be present in the kit. One form in which these instructions may be present is as printed information on a suitable medium or substrate, e.g., a piece or pieces of paper on which the

information is printed, in the packaging of the kit, in a package insert, etc. Yet another means would be a computer readable medium, e.g., diskette, CD, etc., on which the information has been recorded. Yet another means that may be present is a website address which may be used via the internet to access the information at a remote site. Any convenient means may be present in the kits.

Accordingly, in a further embodiment of the invention is provided a kit for detecting or enriching for a target nucleic acid present in a nucleic acid sample, said kit comprising:

(a) an oligonucleotide B, being a single-stranded oligonucleotide which may contain or carry at least one element for detection and/or enrichment (or more particularly detection, amplification and/or capture) of a target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of an oligonucleotide A, wherein said target fragment and said second non-target-specific part are as defined above, and;

(b) one or more oligonucleotides A, being a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part of the single-stranded portion of a target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B, wherein said single-stranded portion and said target fragment are as defined above, and;

(c)

(i) one or more restriction endonucleases, and/or;

(ii) a common nucleic acid adaptor, or a plurality of variants of such an adaptor which differ from each other at a carried molecular tag and/or at the end by which the adaptor anneals to said fragments.

The invention will be further described with reference to the following non-limiting Examples with reference to the following drawings in which:

Figure 1 shows a particular embodiment of the method.

The nucleic acid sample is restriction enzyme digested;

A common double-stranded adaptor is designed to ligate selectively to either the 3' or 5' ends of the fragments. This is achieved by selective phosphorylation/dephosphorylation of the adaptor or the fragments (phosphorylated ends shown as "P");

The fragments are denatured;

The probe is annealed to the target fragment;

The probe is ligated to the target fragment;

Optionally the probe is equipped with a function for immobilization to a solid phase;

The target fragment is amplified using common sequences in the adaptor and oligonucleotide B of the probe.

Figure 2 shows a further particular embodiment of the method.

The nucleic acid sample is restriction enzyme digested;

A portion of either the 5'- or 3'-ends of all fragments are made single-stranded by exonucleolysis, whilst a middle portion remains double-stranded;

The probe is designed to hybridize with one of the two single stranded ends of the target fragment so that a junction susceptible to ligation is created;

The probe is ligated to the target fragment;

The probe is equipped with a function such as a biotin molecule (shown as "B") for solid phase enrichment;

Optionally the target fragment is amplified using a common sequence in oligonucleotide B of the probe.

Figure 3 shows a further particular embodiment of the method.

The sample nucleic acid is fragmented using physical or enzymatic means;

Optionally, a common double-stranded adaptor is ligated specifically to the 3' ends of the fragments by e.g. dephosphorylating the sample and using a phosphorylated adaptor (phosphorylated ends shown as "P");

The fragments are denatured;

The probe is annealed to the target fragment to form a substrate for a flap endonucleolytic cleavage of the target fragment 5' end;

The probe is ligated to the target fragment;

Optionally the probe is equipped with a function for solid phase immobilization and enrichment;

The probe is amplified and or detected using the common sequences in oligonucleotide B of the probe and/or optionally the adaptor.

Figure 4 shows a further particular embodiment of the method.

The target fragment is equipped with a probe according to any of the embodiments of the method of the invention;

The distal part of the target fragment with respect to the probe is made double-stranded where this is not already the case. This may, for example, be

mediated by polymerisation initiated from a hybridised probe 3' end or by annealing of hexamers followed by polymerisation;

The probe is designed to allow intramolecular circularisation with the distal end of the probe-target fragment hybrid molecule, formed by ligation;

The intramolecular ligation may, e.g., be by sticky end ligation or blunt end ligation;

Circularised probe-target fragment hybrids are then enriched for using rolling circle amplification (RCA), hyperbranched RCA, multiple strand displacement, exonucleolysis or other methods that increase the ratio between circular and linear molecules.

Figure 5 shows the amplification products from the amplification reaction. Am3 = amine adaptor ligated to fragments. NoDNA = negative control where no genomic DNA was included in the hybridization reaction. All 17 fragments that were selected are between 200 and 450 nucleotides long.

Figure 6 shows qPCR readout of amine blocked selection. Ct values for each primer pair are shown left to right along the x-axis, corresponding to descending order of the primer pairs listed in the key.

EXAMPLES

Example 1

Restriction enzyme digested human genomic DNA was incubated with T4 DNA polymerase in order to generate blunt ends. Subsequently a 3' adenosine overhang was generated by addition of Taq polymerase and the product was ligated to an adaptor with 3' protruding thymidines in separate reactions using T4 DNA ligase. Seventeen *in silico* designed probe-oligonucleotide As together with a common probe-oligonucleotide B were then added to the reaction mix. The reaction was then heat denatured and the probes were allowed to hybridize to the single stranded ends of 17 specific genomic fragments in the restriction enzyme digested genomic DNA sample. After hybridization the probes were captured by a solid phase (beads) using a biotin on the probe. Following stringent washing probes were ligated on the beads and all fragments were amplified by PCR using one common primer pair located in the probe and adaptor respectively. The results were analyzed by gel electrophoresis and qPCR.

The sequences of the 17 probe-oligonucleotide As, adaptor, probe-oligonucleotide B and primer pair are outlined in Table 1. The "amine" terminology used in connection with the adaptors indicates that one end of the adaptors (the end not intended to ligate with the nucleic acid fragments) is "protected" from such ligation by an amine (NH₂) group.

Table 1

Target ID	Probe-oligonucleotide A
ROI_1 (SEQ ID NO: 1)	ACAACGGGAATTCAAATCAAGATGGTGGCCACACCCCATGCGTACCAATGGATGCGGTCT
ROI_2 (SEQ ID NO: 2)	AAAAGAAGCAGGGAGTCAACTTCCTGCACCTTTAACTCTACGTACCAATGGATGCGGTCT
ROI_3 (SEQ ID NO: 3)	CACCTGATGATGGTCMTGGAGGCATTGTTCTGATTCTTTGCGTACCAATGGATGCGGTCT
ROI_4 (SEQ ID NO: 4)	CACACACCAGACTTGTCAGCCTCCCAAAGAGCCATGCTCCCGTACCAATGGATGCGGTCT
ROI_5 (SEQ ID NO: 5)	CACTGGCTGAGGTGCCAGATGGGTCTCCTGGCTATAGGGACGTACCAATGGATGCGGTCT
ROI_6 (SEQ ID NO: 6)	TCTTCAAAGGCAAGGTTTCAATTATGCAAAAACCACCTTACGTACCAATGGATGCGGTCT
ROI_7 (SEQ ID NO: 7)	AAYCTACGGCCATGGCAGGTAAGACGCTGCAGGGAAGCAGCGTACCAATGGATGCGGTCT
ROI_8 (SEQ ID NO: 8)	TGCTGTTTCCACATAGCAGAGGCTGTTAGGCTCAGAGCTACGTACCAATGGATGCGGTCT
ROI_9 (SEQ ID NO: 9)	GGGGTGCTTGAGAGCTTCGCTTGCACTTATTCGCCAACTACGTACCAATGGATGCGGTCT
ROI_10 (SEQ ID NO: 10)	TCTGTGGTAGTACCTAGAATAAGCTATGCAGCCTCTCTGACGTACCAATGGATGCGGTCT
ROI_11 (SEQ ID NO: 11)	GACAGCCGTGTATTACCCAATATCCCCAAAGGCAGCCTTACGTACCAATGGATGCGGTCT
ROI_12 (SEQ ID NO: 12)	GGTTCTGCTCCTCATCCGCTGTGGATCAGATGTGCTCTGACGTACCAATGGATGCGGTCT
ROI_13 (SEQ ID NO: 13)	AATCTTTTGAACCCAATTGGTCATATTTCTTCTACTTACGTACCAATGGATGCGGTCT
ROI_14 (SEQ ID NO: 14)	TGCTGTTTCTCAAATCCGAACGCTCTGTGATAAATTTCTGCGTACCAATGGATGCGGTCT
ROI_15 (SEQ ID NO: 15)	CTCACAGAAACCAAGCGTTTCCCAACAGCACGTTTCTGCGTACCAATGGATGCGGTCT
ROI_16 (SEQ ID NO: 16)	AATTTTAGAAATCCTTRAATTTTCCATGTCTACATTCATGCGTACCAATGGATGCGGTCT
ROI_17 (SEQ ID NO: 17)	GTTCTATGAAAAGAAAAAAGAAACGATTAAGGTTTCATGCGTACCAATGGATGCGGTCT
Adaptor 5'-3'	
Amine plus (SEQ ID NO: 18)	CGTTATCAACCTGGGTCCGA
Amine minus (SEQ ID NO: 19)	TCGGACCCAGGTTGATAACGT
Probe-oligonucleotide B 5'-3'	
(SEQ ID NO: 20)	CTGGACCTTAATCGTGTGCGAGACCGCATCCATTGGTACG
Common primer pair 5'-3'	
Fwd primer (SEQ ID NO: 21)	AGACCGCATCCATTGGTACG
Rev primer (SEQ ID NO: 22)	TCGGACCCAGGTTGATAACG

Restriction enzyme digestion

Genomic DNA was restriction enzyme digested using restriction 10 different restriction enzymes combined in 5 separate reactions outlined in Table 2. 10 units of each restriction enzyme were used to digest 1 µg of DNA at 37 °C for 2 h.

Table 2

Target ID	Target length (bp)	Restriction reaction	5' end	3' end	qPCR primer pair
ROI_1	314	XmnI/HpyCH4V	blunt	blunt	P01
ROI_2	305	BfaI/AlwI	5'o-h	5'o-h	P02
ROI_3	395	XmnI/HpyCH4V	blunt	blunt	P03
ROI_4	260	HpyAV/DraI	blunt	blunt	P04
ROI_5	386	NspI/MlyI	3'o-h	3'o-h	P05
ROI_6	288	DdeI/BspHI	5'o-h	5'o-h	P06
ROI_7	270	HpyAV/DraI	blunt	blunt	P07
ROI_8	264	BfaI/AlwI	5'o-h	5'o-h	P08
ROI_9	287	BfaI/AlwI	5'o-h	5'o-h	P09
ROI_10	205	DdeI/BspHI	5'o-h	5'o-h	P10
ROI_11	301	DdeI/BspHI	5'o-h	5'o-h	P11
ROI_12	226	DdeI/BspHI	5'o-h	5'o-h	P12
ROI_13	361	DdeI/BspHI	5'o-h	5'o-h	P13
ROI_14	355	XmnI/HpyCH4V	blunt	blunt	P14
ROI_15	226	XmnI/HpyCH4V	blunt	blunt	P15
ROI_16	385	DdeI/BspHI	5'o-h	5'o-h	P16
ROI_17	365	DdeI/BspHI	5'o-h	5'o-h	P17

Table 3 - Primer pairs used

Primer pair	Target	Forward	Reverse
P1	ROI_1	TTCCCCACTGACAGCCTC (SEQ ID NO: 23)	GACCCAAAACCCAAAATGG (SEQ ID NO: 24)
P2	ROI_2	CCCCTGTGGACCTCAACC (SEQ ID NO: 25)	TGCTTGAAAAGCCAGTGC (SEQ ID NO: 26)
P3	ROI_3	TCACGGAGGCATTCTAAAGTC (SEQ ID NO: 27)	TTGATGCCCCCAAGAATC (SEQ ID NO: 28)
P4	ROI_4	CCAAGGGCATCCAGTTTG (SEQ ID NO: 29)	GGGGCCACACATCTTC (SEQ ID NO: 30)
P5	ROI_5	ACCTTCCTTGCCCCTCTG (SEQ ID NO: 31)	TCCAGCCGTCAACTCCTC (SEQ ID NO: 32)
P6	ROI_6	TGAGCAAATCCAGTCAGGG (SEQ ID NO: 33)	ACTGTGTGGCAAAGTGC (SEQ ID NO: 34)
P7	ROI_7	TGAAAAGAGAACATGGGGG (SEQ ID NO: 35)	GAGAAAGCCCTTTCCAGGC (SEQ ID NO: 36)
P8	ROI_8	CTCACCTTTGCGCCTCTG (SEQ ID NO: 37)	GAGGTGGAGAAACGCAGG (SEQ ID NO: 38)
P9	ROI_9	GCGAAGCTCTCAAGCACC (SEQ ID NO: 39)	CATTGAGTCTGGAGTGGAGC (SEQ ID NO: 40)
P10	ROI_10	CTCCTCTGTGCAGGTGGG (SEQ ID NO: 41)	GGATGTCCTCAAGCCGTG (SEQ ID NO: 42)
P11	ROI_11	GGAAACTCCCCTTACCCG (SEQ ID NO: 43)	TGTTGCCATGTCAGCAC (SEQ ID NO: 44)
P12	ROI_12	GTCCCATGGTGCTTGCCAG (SEQ ID NO: 45)	ATCTCTGGCTCCGTCGTG (SEQ ID NO: 46)
P13	ROI_13	TTTGACGGGCATCCTTTC (SEQ ID NO: 47)	ACAGGCGAAGGAGGTGTG (SEQ ID NO: 48)
P14	ROI_14	GTGACCTGCCACCTCCAG (SEQ ID NO: 49)	GCTGGCGTAAAGGTGAGG (SEQ ID NO: 50)
P15	ROI_15	AATGGCAACGACGGGTAG (SEQ ID NO: 51)	CAAACGCTCTGAGACAGCC (SEQ ID NO: 52)
P16	ROI_16	GCCTGGCAGAGCTGAATC (SEQ ID NO: 53)	CCAAGCACCTAACAGGCATC (SEQ ID NO: 54)
P17	ROI_17	CCGAAACATGGATTTGGC (SEQ ID NO: 55)	TCAACCGGCAAAGTCAGC (SEQ ID NO: 56)
G1	Not enriched	GACAGCTCCCCACACACC (SEQ ID NO: 57)	TTCTGCCTGAGCTGACC (SEQ ID NO: 58)
G2	Not enriched	TGCCTCTCTTGCTCTGGG (SEQ ID NO: 59)	GTGGGCATGGGTGAGAAG (SEQ ID NO: 60)
G3	Not enriched	ACAGCTGCCCACTTCTGG (SEQ ID NO: 61)	GCGAGACCAAATCAGG (SEQ ID NO: 62)

G4	Not enriched	ATTCAGGCGCTTTGCATC (SEQ ID NO: 63)	AGGCTGGTCACATGGGTG (SEQ ID NO: 64)
G5	Not enriched	CATGGTCTTGGACTGGGC (SEQ ID NO: 65)	AGCTCGATCTTCATGCGG (SEQ ID NO: 66)
Alu1	Alu repeat	GCGCGGTGGCTCACGCCTGT (SEQ ID NO: 67)	CCTCCCAAAGTGCTGGGATT (SEQ ID NO: 68)
Alu2	Alu repeat	CGCCACTGCACTCCAGCCTG (SEQ ID NO: 69)	CGATCTCCTGACCTCATGAT (SEQ ID NO: 70)

Blunt ending fragments using T4 DNA polymerase

Blunt ends were generated from 0.5 µg of digested genomic DNA using 3 Units of T4 polymerase and 0.1 mM dNTP in 1 × Special Buffer (50 mM KAc, 3 mM MgAc, 20 mM TrisAc, 1 mM DTT, 0.1 µg/µl BSA) in a total reaction volume of 15 µl. Two separate enrichment reactions were performed and one additional negative control reaction without addition of restriction enzyme digested genomic DNA. All reactions were incubated at 12 °C for 15 min and 65 °C for 20 min.

Shrimp alkaline phosphatase (SAP)

5' phosphates were removed by addition of 1 U of SAP in 5 µl 1 × Special Buffer. Reactions were incubated at 37 °C for 15 min and 65 °C for 15 min.

Addition of dATP 3' overhangs

Protruding adenosine 3' ends were generated by addition of 5 U Taq polymerase, 0.5 mM dATP in 5 µl 1 × Special Buffer. Reactions were incubated at 72 °C for 20 min.

TA-ligation to adaptors

Fifteen Units of T4 DNA Ligase was used to ligate 0.83 µM amine modified adaptor (5 × the concentration of all fragment ends) and 1 mM ATP to the genomic DNA. Total reaction volumes were 30 µl in 1 × Special Buffer. Reactions were incubated over night at 16 °C and heat inactivated at 65 °C for 20 min.

Enrichment

Probe hybridizations were performed using 12.5 µl of the 30 µl reactions, 0.1 nM of each probe-oligonucleotide A and 7.73 nM of the biotinylated probe-oligonucleotide B, 27.27 % formamide and 0.7 × Bind&Wash buffer in a total reaction volume of 27.5 µl (1 × B&W: 1 M NaCl, 5 mM EDTA, 0.1 % Tween-20, 10 mM Tris-HCl pH 7.5). Reactions were incubated at:

95 °C for 10 min, 75 °C for 30 min, 68 °C for 30 min, 62 °C for 30 min, 55 °C for 30 min, 46 °C for 10h, 10 °C overnight.

Probes were captured using 10 µg streptavidin-coated magnetic beads (Dynabeads M-280, Invitrogen) in a total volume of 200 µl 1.09 × B&W buffer and 3.8 % Formamide. Reactions were incubated at room temperature for 1 h with rotation.

The supernatant was removed using a magnet rack and reactions were washed with 200 µl 1 × B&W buffer and 20 % formamide at 46 °C for 30 min with rotation.

The washing solution was removed using a magnet rack and beads washed with 200 µl 1 × B&W buffer at 46 °C for 10 min with rotation.

After removing the washing buffer using a magnet rack reactions were phosphorylated and ligated in the same reaction by addition of 5 U PNK, 1 mM ATP, 12.5 U Ampligase, 1 × Ampligase buffer, 1 mM NAD and 0.2 µg/µl BSA in a total volume of 50 µl. Reactions were incubated at 37 °C for 30 min and 55 °C for 1 h.

The ligation reactions were removed using a magnetic rack. PCR was performed using 1.5 U Taq Platinum polymerase, 0.1 µM primers, 0.2 mM of each dATP, dUTP, dCTP and dGTP, 2 mM MgCl₂ and 1 × PCR buffer in a final volume of 50 µl.

PCR reactions were incubated at 95 °C for 3 min followed by 40 cycles at 95 °C for 30 sec, 55 °C for 1 min and 72 °C for 1 min and a final step at 72 °C for 10 min.

Reactions were heated to 95 °C for 5 min and supernatants were transferred to new tubes using a magnet rack.

Five microlitres of the amplified products were analyzed using 1 % ethidium bromide-containing agarose gel electrophoresis and the results are presented in Figure 5.

qPCR was then performed on the enriched fragments. The qPCR analysis used 17 primer pairs, one targeting each of the 17 enriched genomic fragments (P1-17, Table 3) as well as 5 primer pairs amplifying regions that were not enriched (G1-5). Two PCR reactions using primers targeting Alu-repeats were also performed (Alu1-2). 10 µl 1:100 diluted PCR product was used in each reaction with 0.9 Units Taq Platinum polymerase, 0.17 µM primer, 0.2 mM dNTP, 1 × SYBR® Green I, 10 % DMSO and 2 mM MgCl₂ in 1 × PCR buffer to a total volume of 30 µl.

Example 2

Genomic DNA from DNA samples of interest is digested with a rare-cutting restriction enzyme. It may be preferable to use a type IIs enzyme, because thereby restriction fragments will not share end sequences. The enzyme should furthermore leave 5' overhangs in order to be suitable for processing in the subsequent step.

Treat the digested DNA with exonuclease III for a predetermined time, adjusted to remove a suitable length of the 3' end sequence of all restriction fragments.

Add a large amount of probe-oligonucleotide As whose 5' "halves" are complementary to (one of) the 5' ends of the restriction fragments that should be isolated, while the other "halves" of the oligonucleotide As contain a standard sequence. The aim is to reach all copies of the restriction fragments. Preferably each half of the oligonucleotide As encompasses 30 or so nucleotides.

Also add an even greater amount of probe-oligonucleotide Bs complementary to the standard sequence of the oligonucleotide As under step 3, and modified at the 5' ends for efficient capture on a solid phase either before or after being ligated to the genomic restriction fragments.

The digested genomic DNA (step 2) and probe oligonucleotides (steps 3 and 4) are incubated under suitably stringent conditions.

Add DNA ligase and cofactors so that probes now become covalently joined to the appropriate genomic DNA fragments.

If desired, add a strand-displacing polymerase and nucleotides to repair the ExoIII-digested ends of the restriction fragments, also copying the covalently joined oligonucleotides.

If ligation was performed in solution, then free probes can now optionally be removed by a simple size separation step.

If ligation was performed in solution, the probes that were joined to the restriction fragments by ligation are next immobilized on a solid-phase. An attractive mechanism for capture on solid support is through click chemistry, but many alternatives can be contemplated, including via biotin-streptavidin. The nature of the solid phase is also important. Possibly very small particles could be used to efficiently reach all oligonucleotides, and then prevent movement of the modified DNA through a gel. Alternatively, a chromatographic matrix could be extensively modified with groups that can be reacted covalently or non-covalently with ones on oligonucleotide B (step 4).

Separate the particles/matrix from DNA remaining in solution.

Release the captured fragments by digestion with an enzyme designed to cleave the immobilized oligonucleotides, e.g. using a rarely-cutting restriction site included in oligonucleotide B on the solid phase (step 4).

If required the released and fragmented DNA can now be ligated again to give rise to double-stranded DNA circles or linear concatemers that can be amplified by whole-genome amplification procedures. Alternatively the released DNA can be directly applied for sequencing.

Claims

1. A method for detecting or enriching for a target deoxyribonucleic acid (DNA) present in a nucleic acid sample, said method comprising:

(a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target DNA;

(b) non-target-specifically annealing to the ends of said fragments one, or a plurality of variants of a, common nucleic acid adaptor, wherein the annealed adaptor becomes ligated to the fragments at the 3' ends and/or the 5' ends of the strands of the fragments such that an adaptor is ligated only to the end of the strand of the target fragment to which, at the other end, oligonucleotide B of the probe is ligated in step (e), and wherein said variant adaptors differ from each other at a carried molecular tag and/or at the end by which the adaptor anneals to said fragments;

(c) rendering said fragments, including said target fragment, at least partially single-stranded, wherein the single-stranded portion includes an end portion and wherein the length of said single-stranded portion is sufficient to allow hybridisation of at least part of the single-stranded portion of said target fragment to the probe of step (d);

(d) contacting the at least partially single-stranded fragments of step (c) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:

(i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part of said single-stranded portion of said target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and

(ii) oligonucleotide B is a single-stranded oligonucleotide which may contain or carry at least one element for detection and/or enrichment of said target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A, such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment;

(e) directly or indirectly ligating oligonucleotide B of said probe to the part of the single-stranded portion of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid; and

- (f) detecting or enriching for said probe-target fragment hybrid.
2. The method of claim 1 for detecting or enriching for a plurality of target DNAs, wherein in step (d) said target fragments are contacted with oligonucleotides A and B of a plurality of nucleic acid probes, each having an oligonucleotide A with a different first target-specific part, whereby a plurality of different target fragments may be respectively annealed to said probes.
3. The method of claim 2, wherein in said plurality of nucleic acid probes oligonucleotide B comprises a common sequence which is the same in each probe.
4. The method of claim 3, wherein said common sequence in each probe comprises the detection and/or enrichment element.
5. The method of any one of claims 2 to 4, wherein the plurality of nucleic acid probes comprise the same oligonucleotide B.
6. The method of any one of claims 1 to 5, wherein said nucleic acid sample comprises genomic DNA or a subfraction thereof.
7. The method of any one of claims 1 to 5, wherein said nucleic acid sample comprises cDNA or a subfraction thereof.
8. The method of any one of claims 1 to 7, wherein said target fragment comprises at least 30 nucleotides.
9. The method of any one of claims 1 to 8, wherein in step (a) said fragmenting is: (i) enzymatic; and/or (ii) physical.
10. The method of claim 9, wherein said enzymatic fragmenting is by means of a restriction endonuclease.
11. The method of claim 9 or claim 10, wherein said enzymatic fragmenting is by means of an enzyme for which a recognition sequence close to the target DNA in the nucleic acid sample is polymorphic, whereby said detecting or enriching for said target DNA is haplotype-specific.

12. The method of any one of claims 1 to 9, wherein said fragmenting is by subjecting aliquots of said nucleic acid sample to respectively different combinations of one or more enzymatic and/or physical methods, and pooling said aliquots of fragmented nucleic acid sample prior to step (c).
13. The method of any one of claims 1 to 11, wherein in step (c) said rendering at least partially single-stranded is by: (i) denaturation or (ii) exonucleolysis.
14. The method of any one of claims 1 to 12, wherein in step (d) said first target-specific part of oligonucleotide A comprises at least 10 nucleotides complementary in sequence to a single-stranded portion at the end of said at least partially single-stranded target fragment.
15. The method of any one of claims 1 to 14, wherein the length of the first target-specific part of oligonucleotide A in step (d) is at least 20 nucleotides.
16. The method of any one of claims 1 to 15, wherein in step (d) said enrichment element contained or carried by oligonucleotide B is an amplification and/or capture element.
17. The method of claim 16, wherein said amplification and capture elements are, respectively, an amplification primer binding site and an element for immobilisation to a solid phase.
18. The method of any one of claims 1 to 14, wherein in step (d) oligonucleotide B is immobilised to a solid phase.
19. The method of any one of claims 1 to 18, wherein in step (d) oligonucleotide B further carries or contains a molecular tag.
20. The method of any one of claims 1 to 19, wherein in step (e) said ligating of oligonucleotide B is to the end of said target fragment.
21. The method of claim 17, wherein in step (f) said target fragment in said probe-target fragment hybrid is amplified from a primer hybridised to said amplification primer binding site.

22. The method of claim 17, wherein in step (f) said target fragment in said probe-target fragment hybrid is immobilised to a solid phase by means of said immobilisation element.

23. The method of any one of claims 1 to 22, wherein in step (f) said target fragment in said probe-target fragment hybrid, or an amplification product thereof, is sequenced.

24. The method of any one of claims 1 to 23, wherein said common nucleic acid adaptor comprises an element for amplification.

25. The method of claim 24, wherein in step (f) said target fragment in said probe-target fragment hybrid is amplified by means of the amplification elements in oligonucleotide B of the probe and in the adaptor.

26. The method of any one of claims 1 to 23, wherein said common nucleic acid adaptor carries a molecular tag.

27. The method of any one of claims 24 to 26, wherein said non-specific annealing is by rendering the ends of the fragments sticky with respect to at least one end of the adaptor, and wherein said ligating of the adaptor to the fragments at the 3' ends, or at the 5' ends, of the strands of the fragments is by selective dephosphorylation of the fragments or the adaptor, respectively, prior to ligation.

28. The method of any one of claims 24 to 27, wherein in step (a) said fragmenting a nucleic acid sample comprises separately fragmenting a plurality of nucleic acid samples, and step (b) comprises ligating to the fragments of which samples, respectively, variant adaptors carrying different molecular tags and pooling said adaptor-ligated fragmented nucleic acid samples.

29. The method of any one of claims 1 to 28, wherein in step (c) said fragments are rendered partially, but not completely, single-stranded.

30. The method of any one of claims 24 to 27, wherein in step (a) said fragmenting is as defined in claim 10, and step (b) comprises ligating to the fragments of said pooled aliquots variant adaptors which differ at the end by which the adaptor anneals to said fragments.

31. The method of any one of claims 1 to 13 and 15 to 30, wherein in step (d) said first target-specific part of oligonucleotide A comprises at least 10 nucleotides complementary in sequence to a single-stranded internal non-end portion of said at least partially single-stranded target fragment, and wherein said annealing of said probe to said target fragment is through hybridisation of said internal non-end portion to said first target-specific part of oligonucleotide A, causing the 5' end of the target fragment to form a substrate for flap endonucleolytic cleavage and further comprising cleaving said flap endonucleolytic cleavage substrate to produce a ligatable 5' end of said target fragment which is hybridised to oligonucleotide A of said probe.

32. The method of claim 31, wherein in step (e) said ligating of oligonucleotide B is to said ligatable 5' end of said target fragment.

33. The method of any one of claims 1 to 10, 24 and 26 to 32, wherein in step (f) said detecting or enriching is by means that increases the ratio of circular to linear molecules, further comprising, between steps (e) and (f), rendering double-stranded an end of at least the target fragment, being the end to which the probe is not ligated, which end will comprise a common nucleic acid adaptor sequence ligated to the fragments in step (b); non-target-specifically annealing said double-stranded end intramolecularly with the free, non-target fragment-bound end of the probe; and ligating oligonucleotide B of the probe-target fragment hybrid with the corresponding strand of said double-stranded end to circularise said hybrid.

34. The method of claim 33, wherein in step (f) said means that increases the ratio of circular to linear molecules is by exonuclease treatment and/or rolling circle amplification.

35. A method for detecting or enriching for a target deoxyribonucleic acid (DNA) present in a nucleic acid sample, said method comprising:

- (a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target DNA;
- (b) non-target-specifically annealing to the ends of said fragments a common nucleic acid adaptor, wherein the annealed adaptor becomes ligated to the fragments only at the 3' ends, or only at the 5' ends, of the strands of the fragments;
- (c) rendering said fragments, including said target fragment, single-stranded;

(d) contacting the single-stranded fragments of step (c) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:

(i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to a portion at the end of said single-stranded target fragment other than the end to which the common nucleic acid adaptor is ligated in step (b), and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and

(ii) oligonucleotide B is a single-stranded oligonucleotide which contains or carries an amplification primer binding site and optionally an element for immobilisation to a solid phase, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A,

such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment;

(e) ligating oligonucleotide B of said probe to the end of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid; and

(f) enriching for said probe-target fragment hybrid through amplification by means of the amplification primer binding site in oligonucleotide B of the probe and an amplification primer binding site in the common nucleic acid adaptor, and optionally immobilising said fragment to a solid phase by means of the immobilisation element in oligonucleotide B of the probe.

36. A method for detecting or enriching for a target deoxyribonucleic acid (DNA) present in a nucleic acid sample, said method comprising:

(a) fragmenting a nucleic acid to generate nucleic acid fragments including a target fragment containing said target DNA;

(b) rendering said fragments, including said target fragment, partially single-stranded by 3' or 5' exonuclease digestion, wherein the length of the resulting single-stranded end portion is sufficient to allow hybridisation of at least part of the single-stranded end portion of said target fragment to the probe of step (c);

(c) contacting the partially single-stranded fragments of step (b) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:

(i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part of said single-stranded portion of said target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and

(ii) oligonucleotide B is a single-stranded oligonucleotide which contains or carries an element for immobilisation to a solid phase and optionally an amplification primer binding site, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A,

such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment ;

(d) ligating oligonucleotide B of said probe to the end of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid; and

(e) enriching for said probe-target fragment hybrid through immobilisation to a solid phase by means of the immobilisation element in oligonucleotide B of the probe, and optionally by amplifying said target fragment by means of the amplification primer binding site in oligonucleotide B of the probe.

37. A method for detecting or enriching for a target deoxyribonucleic acid (DNA) present in a nucleic acid sample, said method comprising:

(a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target DNA;

(b) optionally, non-target-specifically annealing to the ends of said fragments a common nucleic acid adaptor, wherein the annealed adaptor becomes ligated to the fragments at the 3' ends or at the 3' and 5' ends of the strands of the fragments such that an adaptor is ligated to at least the 3' end of the strand of the target fragment to which, at the 5' end, oligonucleotide B of the probe is ligated in step (f);

(c) rendering said fragments, including said target fragment, single-stranded;

(d) contacting the single-stranded fragments of step (c) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:

- (i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to an internal non-end portion of said single-stranded target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and
- (ii) oligonucleotide B is a single-stranded oligonucleotide which contains or carries at least one element for detection and/or enrichment of said target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A, such that said target fragment becomes annealed at said internal non-end portion to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment, wherein said hybridisation causes the 5' end of the target fragment to form a substrate for flap endonucleolytic cleavage;
- (e) cleaving said flap endonucleolytic cleavage substrate to produce a ligatable 5' end of said target fragment which is hybridised to oligonucleotide A of said probe;
- (f) ligating oligonucleotide B of said probe to the part of the single-stranded portion of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid;
- (g) enriching for said probe-target fragment hybrid by means of said detection and/or enrichment element.

38. A method for detecting or enriching for a target deoxyribonucleic acid (DNA) present in a nucleic acid sample, said method comprising:

- (a) fragmenting a nucleic acid sample to generate nucleic acid fragments including a target fragment containing said target DNA;
- (b) optionally, non-target-specifically annealing to the ends of said fragments a common nucleic acid adaptor, wherein the annealed adaptor becomes ligated to the fragments at the 3' ends and/or at the 5' ends of the strands of the fragments such that an adaptor is ligated only to the end of the strand of the target fragment to which, at the other end, oligonucleotide B of the probe is ligated in step (e);
- (c) rendering said fragments, including said target fragment, at least partially single-stranded, wherein the single-stranded portion includes an end portion and wherein the length of said single-stranded portion is sufficient to allow

hybridisation of at least part of the single-stranded portion of said target fragment to the probe of step (d);

(d) contacting the at least partially single-stranded fragments of step (c) with oligonucleotides A and B of a single target-specific nucleic acid probe, wherein:

(i) oligonucleotide A is a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part of said single-stranded portion of said target fragment, wherein if a common nucleic acid adaptor is ligated to the fragments according to optional step (b) said first target-specific part comprises at least 10 nucleotides complementary in sequence to a portion at the end of said at least partially single-stranded target fragment other than the end to which the common nucleic acid adaptor is ligated;

and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B of the probe, and

(ii) oligonucleotide B is a single-stranded oligonucleotide which contains or carries at least one element for detection and/or enrichment of said target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of oligonucleotide A, such that said target fragment becomes annealed to said probe through hybridisation to the first target-specific part of oligonucleotide A resulting in only one target-specific probe-binding event per target fragment;

(e) ligating oligonucleotide B of said probe to the part of the single-stranded portion of said target fragment which is hybridised to oligonucleotide A of said probe to produce a probe-target fragment hybrid;

(f) if necessary, rendering double-stranded an end of at least the target fragment, being the end to which the probe is not ligated, which end will comprise a common nucleic acid adaptor sequence in the case that such an adaptor is ligated to the fragments according to optional step (b);

(g) non-target-specifically annealing the double-stranded end of step (f) intramolecularly with the free, non-target fragment-bound end of the probe;

(h) ligating oligonucleotide B of the probe-target fragment hybrid with the corresponding strand of the double-stranded end of step (f) to circularise said hybrid; and

(i) enriching for said probe-target fragment hybrid by means that increases the ratio of circular to linear molecules.

39. A kit for detecting or enriching for a target DNA present in a nucleic acid sample, said kit comprising:

(a) an oligonucleotide B, being a single-stranded oligonucleotide which may contain or carry at least one element for detection and/or enrichment of a target fragment, and of which at least a portion, including one end, is complementary in sequence to the second non-target-specific part of an oligonucleotide A, wherein said target fragment and said second non-target-specific part are as defined in claim 1, and;

(b) one or more oligonucleotides A, being a single-stranded oligonucleotide comprising at one end a first target-specific part comprising at least 10 nucleotides complementary in sequence to at least part of the single-stranded portion of a target fragment, and comprising at the other end a second non-target-specific part which comprises a nucleotide sequence complementary to at least a portion, including one end, of oligonucleotide B, wherein said single-stranded portion and said target fragment are as defined in claim 1, and;

(c)

(i) one or more restriction endonucleases; and/or

(ii) a common nucleic acid adaptor, or a plurality of variants of such an adaptor which differ from each other at a carried molecular tag and/or at the end by which the adaptor anneals to said fragments.

Figure 1

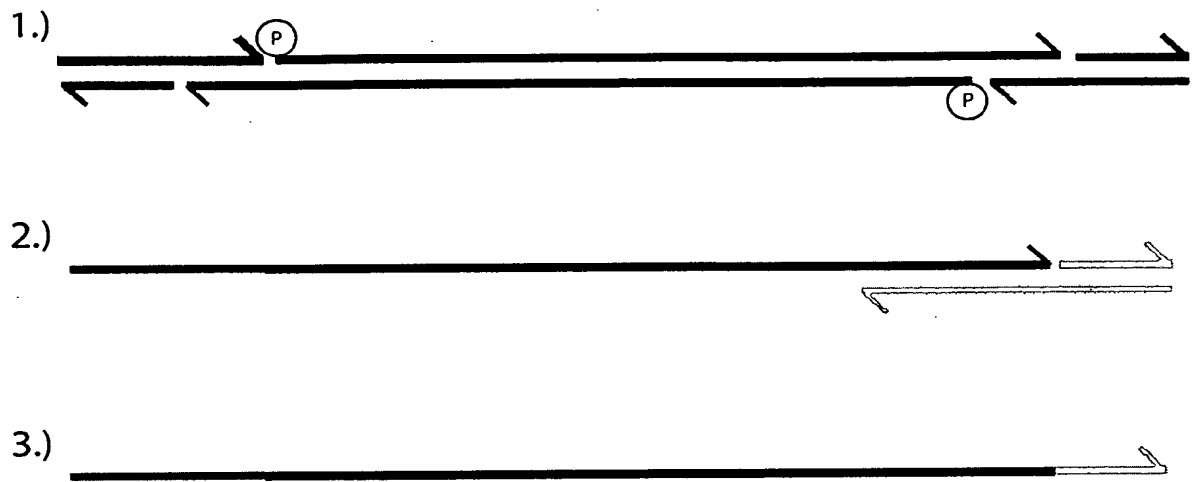
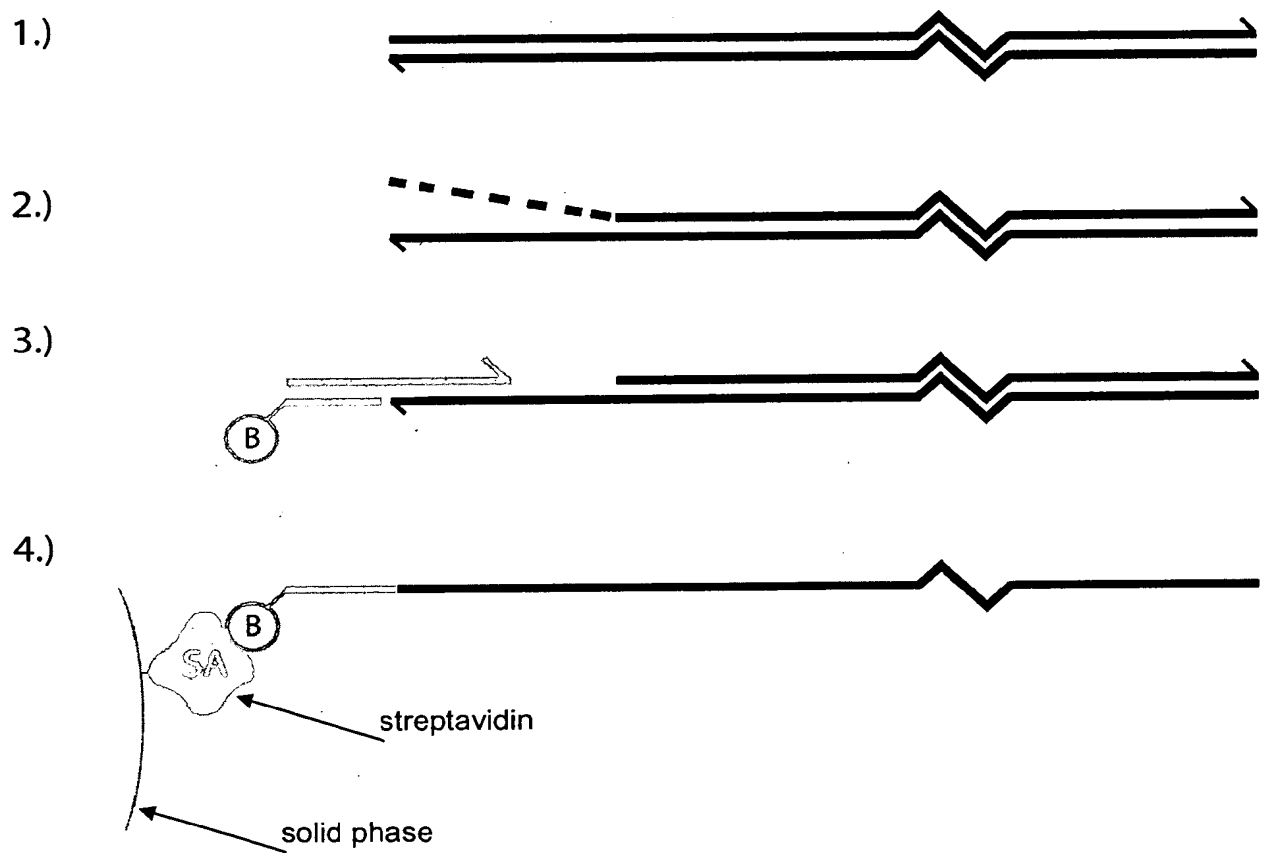


Figure 2



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Figure 3

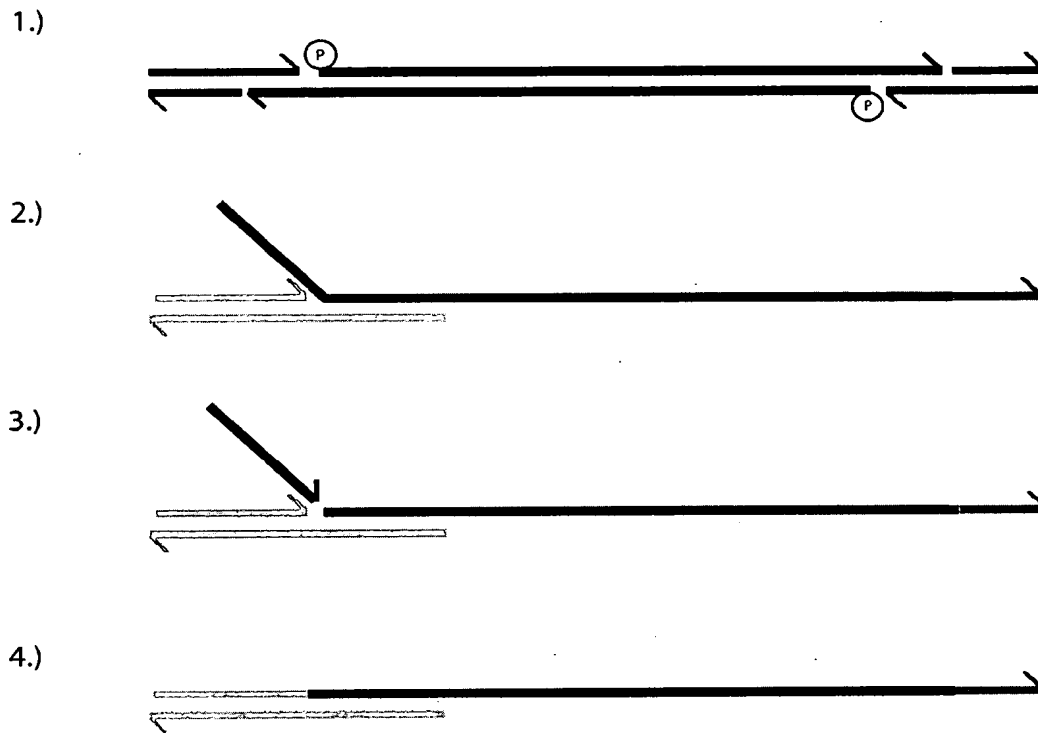
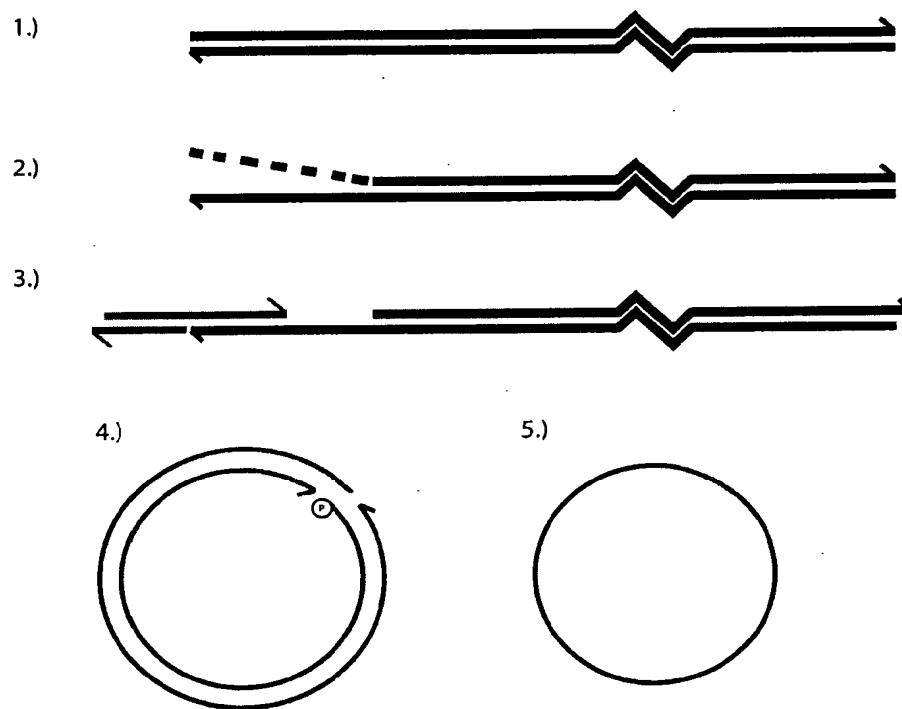


Figure 4



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Figure 5

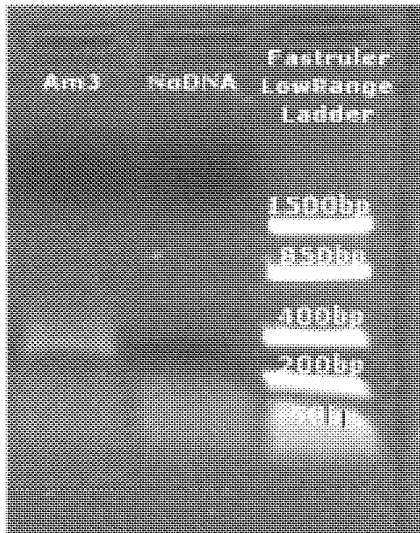
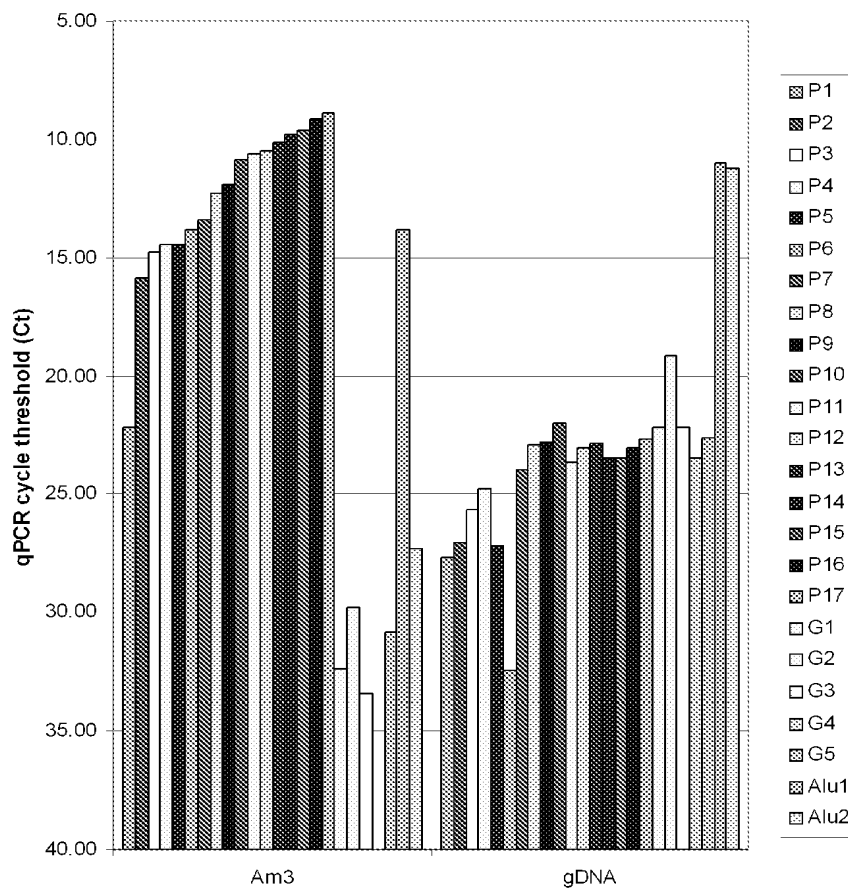


Figure 6



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/060715

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/68

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According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	WO 2005/111236 A1 (OLINK AB [SE]; DAHL FREDRIK [SE]) 24 November 2005 (2005-11-24) the whole document	1-39
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Application Number	16017340
Filing Date	2018-06-25
First Named Inventor	Douglas A. Amorese
Art Unit	1637
Examiner Name	A. M. Bertagna
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Attorney Docket Number	NUGN-037/04US 34079/440

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	2	Callow, 2004, Selective DNA amplification from complex genomes using universal double-sided adaptors, Nucl Acids Res 32(2):e21/1-6	
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Examiner Name	A. M. Bertagna
Attorney Docket Number	NUGN-037/04US 34079/440

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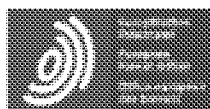
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COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION

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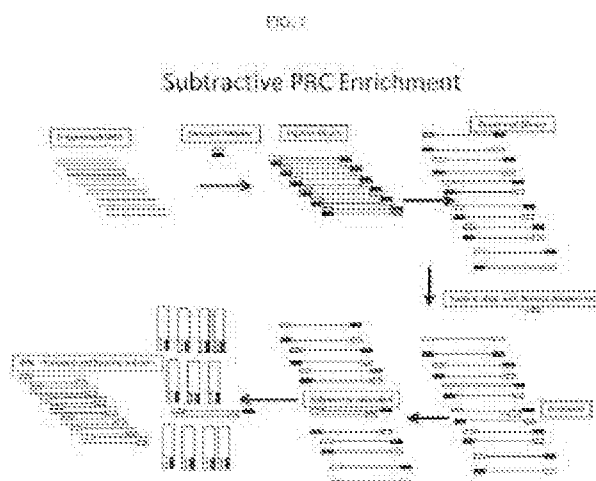
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Abstract of WO2013112923 (A1)

The present invention provides methods, compositions and kits for targeted nucleic acid sequence enrichment in a nucleic acid sample and for high efficiency nucleic acid library generation for next generation sequencing (NGS). Specifically, the methods, compositions and kits provided herein are useful for the production and capture of amplification-ready, target-specific and strand-specific regions of interest from nucleic acid samples containing complex DNA.



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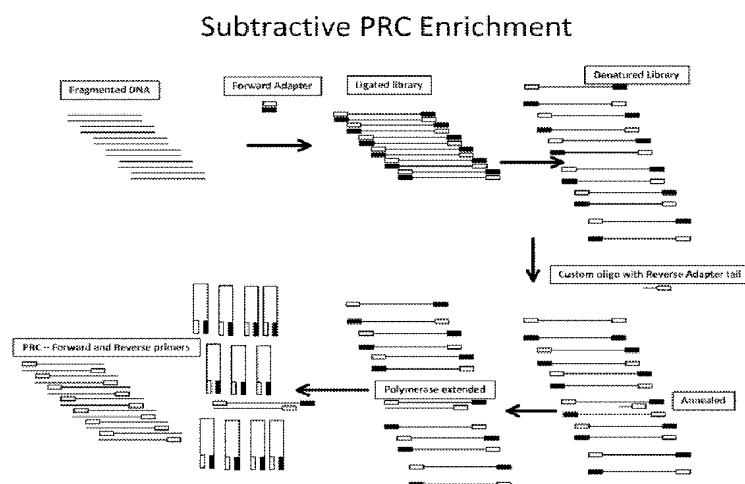
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FIG. 1



(57) Abstract: The present invention provides methods, compositions and kits for targeted nucleic acid sequence enrichment in a nucleic acid sample and for high efficiency nucleic acid library generation for next generation sequencing (NGS). Specifically, the methods, compositions and kits provided herein are useful for the production and capture of amplification-ready, target-specific and strand-specific regions of interest from nucleic acid samples containing complex DNA.

COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 61/591,241, filed January 26, 2012, which application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] With the rapid development of next generation sequencing (NGS) technologies and platforms, whole genome sequencing is becoming increasingly feasible. Researchers are driven to generate increasing amounts of data to achieve greater understanding of variance and biological trends, and to generate data from smaller sample sizes to avoid averaging across multiple cells within a tissue.

[0003] Although the cost of whole genome sequencing is decreasing and the throughput of the NGS platforms is increasing, it is nonetheless often more practical and cost-effective to select genomic regions of interest for sequencing and analysis. Target enrichment is a commonly employed strategy in genomic DNA sequencing in which genomic regions of interest are selectively captured from a DNA sample before sequencing. Focused target enrichment is an important tool especially in the fields of study where sequencing of a large number of samples is necessary (e.g. population-based studies of disease markers or SNPs), making whole genome sequencing cost-prohibitive. Similarly, improvements have been made that enable DNA libraries to be made from nucleic acid from fewer number of cells, but these are bound by the limitations of the efficiency of ligation reactions.

[0004] Several approaches to target enrichment have been developed which vary from one another in terms of sensitivity, specificity, reproducibility, uniformity, cost and ease of use. The target enrichment methods commonly employed today can be divided into three major categories, each with its distinct advantages and disadvantages: 1) PCR-based methods; 2) capture-by-hybridization, i.e. on-array or in-solution hybrid capture; and 3) capture-by-circularization, i.e. molecular inversion probe-based methods.

[0005] The PCR-based methods employ highly parallel PCR amplification, where each target sequence in the sample has a corresponding pair of unique, sequence-specific primers. The simultaneous use of numerous primer pairs makes multiplex PCR

impractical due to high level of non-specific amplification and primer-primer interactions. Recently developed microdroplet PCR technology (Tewhey *et al.*, 2009) in which each amplification reaction is physically separated into an individual droplet removes the constraints of multiplex PCR relating to non-specific amplification and primer-primer interactions. However, microdroplet PCR and other improved PCR-based methods require special instrumentation or platforms, are limited in their throughput, and, as with conventional multiplex PCR, require a large number of individual primer pairs when enriching for a multitude of regions of interest, thus making target enrichment costly.

[0006] Hybrid capture methods are based on the selective hybridization of the target genomic regions to user-designed oligonucleotides. The hybridization can be to oligonucleotides immobilized on high or low density microarrays (on-array capture), or solution-phase hybridization to oligonucleotides modified with a ligand (e.g. biotin) which can subsequently be immobilized to a solid surface, such as a bead (in-solution capture). The hybrid capture methods require complex pools of costly long oligonucleotides and long periods (typically 48 hours) of hybridization for efficient capture. For on-array hybrid capture, expensive instrumentation and hardware is also required. Because of the relatively low efficiency of the hybridization reaction, large quantities of input DNA are needed.

[0007] The molecular inversion probe (MIP) based method relies on construction of numerous single-stranded linear oligonucleotide probes, consisting of a common linker flanked by target-specific sequences. Upon annealing to a target sequence, the probe gap region is filled via polymerization and ligation, resulting in a circularized probe. The circularized probes are then released and amplified using primers directed at the common linker region. One of the main disadvantages of the MIP-based target enrichment is its relatively low capture uniformity, meaning there is large variability in sequence coverage across the target regions. As with PCR and hybrid capture, the MIP-based method requires a large number of target-specific oligonucleotides, which can be costly.

[0008] There is a need for improved methods for selective target enrichment that allow for low-cost, high throughput capture of genomic regions of interest without specialized instrumentation. Additionally, there is also a need for high efficiency nucleic acid library generation. The methods of the invention described herein fulfill these needs.

SUMMARY OF THE INVENTION

[0009] In one aspect, disclosed herein are methods for enriching for target nucleic acid sequences of interest in a sample comprising nucleic acids, the method comprising: (a) fragmenting the nucleic acids, thereby generating nucleic acid fragments; (b) appending a first adaptor to a 5' end of each nucleic acid fragment; (c) annealing one or more oligonucleotides to the nucleic acid fragments, whereby each of the one or more oligonucleotides comprise a 3' portion that is complementary to a target nucleic acid sequence of interest present in one or more of the nucleic acid fragments, and a 5' portion comprising a second adaptor sequence; (d) extending the one or more oligonucleotides with a polymerase thereby generating one or more oligonucleotide extension products with the first adaptor at a first end and the second adaptor sequence at a second end; and (e) amplifying the one or more oligonucleotide extension product using a first primer that is complementary to the first adaptor and a second primer that is complementary to the second adaptor sequence to enrich for nucleic acid fragments containing the first adaptor and the second adaptor sequence at each end. In one embodiment, the method further comprises an additional step of sequencing the one or more oligonucleotide extension product following amplification. In one embodiment, the target nucleic acid sequences of interest comprise genomic DNA, RNA, or cDNA. In one embodiment, the target nucleic acid sequences of interest comprise genomic DNA. In one embodiment, the target nucleic acid sequences of interest comprise cDNA. In one embodiment, the method further comprises denaturing the nucleic acid fragments prior to step c, thereby generating single-stranded nucleic acid fragments with the first adaptor sequence at the 5' end. In one embodiment, the first adaptor can be common to each nucleic acid fragment. In one embodiment, the second adaptor sequence can be common to the one or more oligonucleotides. In one embodiment, the first adaptor and the second adaptor sequence can be distinct from each other. In one embodiment, the first adaptor and/or the second adaptor sequence further comprise barcode sequence. In one embodiment, step b can be performed by ligation. In one embodiment, the method further comprises an additional step of performing gap repair following ligation of the first adapter to create nucleic acid fragments with complementary termini. In one embodiment, a composition comprising enriched target nucleic acid sequences of interest can be generated by the methods disclosed herein. In one embodiment, the polymerase can be a DNA polymerase.

[0010] In another aspect, disclosed herein are methods for enriching for target nucleic acid sequences of interest in a sample comprising nucleic acids, the method comprising: (a) fragmenting the nucleic acids, thereby generating nucleic acid fragments; (b) appending a first adaptor to the nucleic acid fragments wherein the first adaptor comprises a partial duplex with a short strand and a long strand wherein a 3' end of the short strand of the partial duplex adaptor comprises a blocking group, and a 5' end of the long strand of the partial duplex adaptor comprises a restriction and/or cleavage site for a nucleic acid modifying enzyme; (c) denaturing the nucleic acid fragments, thereby creating single-stranded nucleic acid fragments; (d) annealing one or more oligonucleotides to the single stranded nucleic acid fragments, whereby each of the one or more oligonucleotides comprise sequence that is complementary to a target nucleic acid sequence of interest present in one or more of the single-stranded nucleic acid fragments, extending the one or more oligonucleotides with a polymerase to produce one or more double stranded nucleic acid complexes comprising the target nucleic acid sequences and their complements, a first end with a double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme, and a second end with a 3' overhang comprising the short strand of the first adaptor; (e) cleaving the double stranded restriction and/or cleavage site with the nucleic acid modifying enzyme, thereby generating a cleavage site; (f) ligating a second adaptor to the cleavage site, wherein the second adaptor comprises a duplex with two strands; (g) denaturing the one or more double-stranded nucleic acid complexes, thereby generating one or more single stranded nucleic acid fragments comprising the target nucleic acid sequences, a strand from the second adapter at the first end, and the short strand of the first adaptor at the second end; and (h) amplifying the one or more single stranded nucleic acid fragments comprising the one or more target nucleic acid sequences with a first primer comprising sequence complementary to the strand from the second adapter and a second primer comprising sequence complementary to the short strand of the first adaptor, thereby enriching for the one or more target nucleic acid sequences. In one embodiment, the method further comprises an additional step of sequencing the one or more single stranded nucleic acid fragments from step h following amplification. In one embodiment, the target nucleic acid sequences of interest comprise genomic DNA, RNA, or cDNA. In one embodiment, the target nucleic acid sequences of interest comprise genomic DNA. In one embodiment, the target nucleic acid sequences of interest comprise cDNA. In one embodiment, the first adaptor and the second adaptor can

be common to each of the nucleic acid fragments. In one embodiment, the first adaptor and the second adaptor can be distinct from each other. In one embodiment, the first adaptor and/or the second adaptor further comprise barcode sequence. In one embodiment, the double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme from step e comprises the 5' end of the long strand of the partial duplex of the first adaptor and sequence complementary to the 5' end of the long strand of the partial duplex of the first adaptor generated from extension of the one or more oligonucleotides. In one embodiment, the nucleic acid modifying enzyme comprises a restriction enzyme. In one embodiment, step b can be performed by ligation. In one embodiment, a composition comprising enriched target nucleic sequences of interest can be generated by the methods disclosed herein. In one embodiment, the polymerase can be a DNA polymerase.

[0011] In yet another aspect, disclosed herein are methods for generating a library of nucleic acid sequences, the method comprising: (a) fragmenting a sample comprising nucleic acids, thereby generating nucleic acid fragments; (b) appending a first adaptor to each of the nucleic acid fragments; (c) denaturing the nucleic acid fragments, thereby generating a library of single-stranded nucleic acid fragments; (d) annealing one or more oligonucleotides to the single-stranded nucleic acid fragments wherein each of the one or more oligonucleotides comprises a 3' portion complementary to sequence in one or more of the single-stranded nucleic acid fragments and a 5' portion comprising a second adaptor sequence; (e) extending the one or more oligonucleotides with a polymerase thereby generating one or more oligonucleotide extension products comprising the first adaptor at a first end and the second adapter sequence at a second end; and (f) amplifying the one or more oligonucleotide extension products with a set of primers specific to the first adaptor and the second adaptor sequence to generate a library of nucleic acid fragments comprising the first adaptor and second adaptor sequence at each end. In one embodiment, the method further comprises an additional step of performing a gap repair reaction following ligation of the forward adapter to create nucleic acid fragments with complementary termini. In one embodiment, the method further comprises an additional step of sequencing the amplified one or more oligonucleotide extension product from step f. In one embodiment, the nucleic acid sequence comprises genomic DNA. In one embodiment, the nucleic acid sequence comprises cDNA. In one embodiment, step c can be omitted wherein the nucleic acid fragments are double-stranded. In one embodiment,

the 3' portion of the one or more oligonucleotides of step d comprises random sequence. In one embodiment, step b can be performed by ligation. In one embodiment, the first adaptor and the second adaptor sequence can be common to each nucleic acid fragment. In one embodiment, the first adaptor and the second adaptor sequence can be distinct from each other. In one embodiment, the first adaptor and/or the second adaptor sequence further comprise barcode sequence. In one embodiment, the methods disclosed herein can generate a composition comprising a library of nucleic sequences. In one embodiment, the polymerase can be a DNA polymerase.

[0012] In further aspect, disclosed herein are methods for enriching for target nucleic acid sequences of interest from a library comprising nucleic acid inserts with a first adaptor on a first end and a second adaptor on a second end, the method comprising: (a) denaturing the nucleic acid inserts, thereby generating a library of single stranded nucleic acid inserts; (b) annealing one or more oligonucleotides to the single stranded nucleic acid inserts, wherein each of the one or more oligonucleotides comprises a 3' portion that is complementary to a target nucleic acid sequence of interest present in one or more of the nucleic acid inserts, and a 5' portion comprising a third adaptor sequence; (c) extending the one or more oligonucleotides with a polymerase thereby generating one or more oligonucleotide extension products with the first adaptor at the first end and the third adaptor sequence at the second end; and (d) amplifying the one or more oligonucleotide extension products using a first primer that is complementary to the first adaptor and a second primer that is complementary to the third adaptor sequence to enrich for nucleic acid fragments containing the first adaptor and the third adaptor sequence at each end. In one embodiment, the method further comprises an additional step of sequencing the amplified one or more oligonucleotide extension products from step d. In one embodiment, the target nucleic acid sequences of interest comprise genomic DNA. In one embodiment, the target nucleic acid sequences of interest comprise cDNA. In one embodiment, step a can be omitted wherein the nucleic acid fragments can be double-stranded. In one embodiment, the first adaptor and the second adaptor can be common to each nucleic acid fragment. In one embodiment, the third adaptor sequence can be common to the one or more oligonucleotides. In one embodiment, the first adaptor and the second adaptor can be distinct from each other. In one embodiment, the first adaptor and the second adaptor can be the same. In one embodiment, the first adaptor, the second adaptor and/or the third adaptor sequence further comprise barcode sequence. In one

embodiment, the methods disclosed herein can generate a composition comprising enriched target nucleic acid sequences of interest.

INCORPORATION BY REFERENCE

[0013] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0015] FIG. 1 depicts selective target enrichment using ligation of a single forward adaptor at the ends of the DNA fragments in the DNA library. The sequence-specific oligonucleotide that anneals to the target region of interest contains a common reverse adaptor sequence at its 5' end, and following sequence-specific oligonucleotide extension, PCR is performed using a set of primers specific to the forward and reverse adaptors.

[0016] FIG. 2 depicts an alternative ligation protocol wherein the DNA-fragment-adaptor complex is denatured following ligation without nick repair and adaptor fill-in, generating ligation products where non-complementary ends exist on each insert.

[0017] FIG. 3 depicts selective target enrichment using ligation of partial duplex adaptors. Cleavage of the 5' end of the long strand of the partial duplex adaptor (and the corresponding complementary sequence of the extended sequence-specific oligonucleotide) by a nucleic acid modifying enzyme specific for double-stranded DNA allows for ligation of a new adaptor pair, and consequently, amplification with primers corresponding to the new adaptors.

[0018] FIG. 4 depicts high efficiency NGS library generation using random priming. The oligonucleotide that anneals to the DNA fragment contains a common reverse adaptor sequence at its 5' end, and following primer extension, PCR is performed using a set of primers specific to the forward and reverse adaptors.

DETAILED DESCRIPTION OF THE INVENTION

General

[0019] The methods of the invention can be used for the selective enrichment of a plurality of defined target sequences from complex DNA with a set of common primers and adaptors, thus circumventing the need for multiplex PCR and multiple primer pairs. A multiplicity of target regions of interest are envisioned: for example, the regions of interest can represent all known coding regions, the entire exome, selected regions of coding genomic regions representing selected pathways, selected genomic regions known to comprise genomic variation related to altered phenotype, entire or selected regions of a specific chromosome, and the like. In another aspect, the methods of the invention can be used for high efficiency nucleic acid library production as well.

[0020] Altogether, the methods of the present invention create a simple, low cost, high throughput system for target enrichment and library preparation.

[0021] Reference will now be made in detail to exemplary embodiments of the invention. While the disclosed methods and compositions will be described in conjunction with the exemplary embodiments, it will be understood that these exemplary embodiments are not intended to limit the invention. On the contrary, the invention is intended to encompass alternatives, modifications and equivalents, which may be included in the spirit and scope of the invention.

[0022] In one embodiment, the present invention provides methods and compositions for the enrichment of specific target sequences of interest from a sample comprising nucleic acids. The methods described herein enrich target sequences using conventional duplex adaptors and/or partial duplex adaptors, sequence specific oligonucleotides, restriction enzymes and ligation. The methods further enable enrichment of target sequences from specific strands of template nucleic acids which can be further amplified using a variety of amplification methods. In another embodiment, the present invention provides methods for high efficiency generation of libraries comprising specific nucleic acid sequences of interest.

[0023] In one embodiment, the present invention provides methods and compositions for the enrichment of target nucleic acid sequences from a sample comprising nucleic acids. In one aspect, the method comprises fragmenting nucleic acids in an input sample to generate nucleic acid fragments. The nucleic acids can be DNA, or RNA. The nucleic acids can be single or double stranded. The DNA can be genomic DNA or cDNA or any

combination thereof. In one embodiment, the nucleic acids in an input sample are double stranded DNA. In one embodiment, fragmentation of the nucleic acids can be achieved through methods known in the art. Fragmentation can be through physical fragmentation methods and/or enzymatic fragmentation methods. Physical fragmentation methods can include nebulization, sonication, and/or hydrodynamic shearing. In some embodiments, the fragmentation can be accomplished mechanically comprising subjecting the nucleic acids in the input sample to acoustic sonication. In some embodiments, the fragmentation comprises treating the nucleic acids in the input sample with one or more enzymes under conditions suitable for the one or more enzymes to generate double-stranded nucleic acid breaks. Examples of enzymes useful in the generation of nucleic acid or polynucleotide fragments include sequence specific and non-sequence specific nucleases. Non-limiting examples of nucleases include DNase I, Fragmentase, restriction endonucleases, variants thereof, and combinations thereof. Reagents for carrying out enzymatic fragmentation reactions are commercially available (e.g, from New England Biolabs). For example, digestion with DNase I can induce random double-stranded breaks in DNA in the absence of Mg^{++} and in the presence of Mn^{++} . In some embodiments, fragmentation comprises treating the nucleic acids in the input sample with one or more restriction endonucleases. Fragmentation can produce fragments having 5' overhangs, 3' overhangs, blunt ends, or a combination thereof. In some embodiments, such as when fragmentation comprises the use of one or more restriction endonucleases, cleavage of sample polynucleotides leaves overhangs having a predictable sequence. In some embodiments, the method includes the step of size selecting the fragments via standard methods known in the art such as column purification or isolation from an agarose gel.

[0024] In some embodiments, the nucleic acids in the input sample can be fragmented into a population of fragmented nucleic acid molecules or polynucleotides of one or more specific size range(s). In some embodiments, the fragments have an average length from about 10 to about 10,000 nucleotides. In some embodiments, the fragments have an average length from about 50 to about 2,000 nucleotides. In some embodiments, the fragments have an average length from about 100-2,500, 10-1,000, 10-800, 10-500, 50-500, 50-250, or 50-150 nucleotides. In some embodiments, the fragments have an average length less than 10,000 nucleotide, such as less than 5,000 nucleotides, less than 2,500 nucleotides, less than 2,500 nucleotides, less than 1,000 nucleotides, less than 500

nucleotides, such as less than 400 nucleotides, less than 300 nucleotides, less than 200 nucleotides, or less than 150 nucleotides.

[0025] In one embodiment, fragmentation of the nucleic acids can be followed by end repair of the nucleic acid fragments. End repair can include the generation of blunt ends, non-blunt ends (i.e sticky or cohesive ends), or single base overhangs such as the addition of a single dA nucleotide to the 3'-end of the nucleic acid fragments, by a polymerase lacking 3'-exonuclease activity. End repair can be performed using any number of enzymes and/or methods known in the art including, but not limited to, commercially available kits such as the EncoreTM Ultra Low Input NGS Library System I. In a preferred embodiment, end repair can be performed on double stranded DNA fragments to produce blunt ends wherein the double stranded DNA fragments contain 5' phosphates and 3' hydroxyls. In some embodiments, the double-stranded DNA fragments can be blunt-end polished (or "end repaired") to produce DNA fragments having blunt ends, prior to being joined to adapters. Generation of the blunt ends on the double stranded fragments can be generated by the use of a single strand specific DNA exonuclease such as for example exonuclease 1, exonuclease 7 or a combination thereof to degrade overhanging single stranded ends of the double stranded products. Alternatively, the double stranded DNA fragments can be blunt ended by the use of a single stranded specific DNA endonuclease, for example, but not limited to, mung bean endonuclease or S1 endonuclease. Alternatively, the double stranded products can be blunt ended by the use of a polymerase that comprises single stranded exonuclease activity such as for example T4 DNA polymerase, or any other polymerase comprising single stranded exonuclease activity or a combination thereof to degrade the overhanging single stranded ends of the double stranded products. In some cases, the polymerase comprising single stranded exonuclease activity can be incubated in a reaction mixture that does or does not comprise one or more dNTPs. In other cases, a combination of single stranded nucleic acid specific exonucleases and one or more polymerases can be used to blunt end the double stranded fragments generated by fragmenting the sample comprising nucleic acids. In still other cases, the nucleic acid fragments can be made blunt ended by filling in the overhanging single stranded ends of the double stranded fragments. For example, the fragments may be incubated with a polymerase such as T4 DNA polymerase or Klenow polymerase or a combination thereof in the presence of one or more dNTPs to fill in the single stranded portions of the double stranded fragments. Alternatively, the double

stranded DNA fragments can be made blunt by a combination of a single stranded overhang degradation reaction using exonucleases and/or polymerases, and a fill-in reaction using one or more polymerases in the presence of one or more dNTPs.

[0026] In some embodiments, the 5' and/or 3' end nucleotide sequences of fragmented nucleic acids are not modified or end-repaired prior to ligation with the adapter oligonucleotides of the present invention. For example, fragmentation by a restriction endonuclease can be used to leave a predictable overhang, followed by ligation with one or more adapter oligonucleotides comprising an overhang complementary to the predictable overhang on a nucleic acid fragment. In another example, cleavage by an enzyme that leaves a predictable blunt end can be followed by ligation of blunt-ended nucleic acid fragments to adapter oligonucleotides comprising a blunt end. In some embodiments, end repair can be followed by an addition of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides, such as one or more adenine, one or more thymine, one or more guanine, or one or more cytosine, to produce an overhang. Nucleic acid fragments having an overhang can be joined to one or more adapter oligonucleotides having a complementary overhang, such as in a ligation reaction. For example, a single adenine can be added to the 3' ends of end repaired DNA fragments using a template independent polymerase, followed by ligation to one or more adapters each having a thymine at a 3' end. In some embodiments, adapter oligonucleotides can be joined to blunt end double-stranded nucleic acid fragments which have been modified by extension of the 3' end with one or more nucleotides followed by 5' phosphorylation. In some cases, extension of the 3' end can be performed with a polymerase such as for example Klenow polymerase or any of the suitable polymerases provided herein, or by use of a terminal deoxynucleotide transferase, in the presence of one or more dNTPs in a suitable buffer containing magnesium. In some embodiments, nucleic acid fragments having blunt ends can be joined to one or more adapters comprising a blunt end. Phosphorylation of 5' ends of nucleic acid fragments can be performed for example with T4 polynucleotide kinase in a suitable buffer containing ATP and magnesium. The fragmented nucleic acid molecules may optionally be treated to dephosphorylate 5' ends or 3' ends, for example, by using enzymes known in the art, such as phosphatases.

[0027] The methods described herein for enriching for target nucleic acid sequences further comprise appending a first adaptor to the nucleic acid fragments generated by the methods described herein. In one embodiment, the first adaptor can be a forward adaptor.

Appending the first adaptor to the nucleic acid fragments generated by methods described herein can be achieved using a ligation reaction or a priming reaction. In one embodiment, appendage of a first adaptor to the nucleic acid fragments comprises ligation. In one embodiment, ligation of the first adaptor to the nucleic acid fragments can be following end repair of the nucleic acid fragments. In another embodiment, the ligation of the first adaptor to the nucleic acid fragments can be following generation of the nucleic acid fragments without end repair of the nucleic acid fragments. The first adaptor can be any type of adaptor known in the art including, but not limited to, conventional duplex or double stranded adaptors in which the adaptor comprises two complementary strands. In a preferred embodiment, the first adaptor can be a double stranded DNA adaptor. In one embodiment, the first adaptor can be an oligonucleotide of known sequence and, thus, allow generation and/or use of sequence specific primers for amplification and/or sequencing of any polynucleotides to which the first adaptor(s) is appended or attached. In one embodiment, the first adaptor can be a conventional duplex adaptor, wherein the first adaptor comprises sequence well known in the art. In a preferred embodiment, the first adaptor can be appended to the nucleic acid fragments generated by the methods described herein in multiple orientations. In a preferred embodiment, the methods described herein can involve the use of a first duplex adaptor comprising double stranded DNA of known sequence that is blunt ended and can bind to the double stranded nucleic acid fragments generated by the methods described herein in one of two orientations. In one embodiment, the first adaptor can be ligated to each of the nucleic acid fragments such that each of the nucleic acid fragments comprises the same first adaptor. In other words, each of the nucleic acid fragments comprises a common first adaptor. In another embodiment, a first adaptor can be appended or ligated to a library of nucleic acid fragments generated by the methods described herein such that each nucleic acid fragment in the library of nucleic acid fragments comprises the first adaptor ligated to one or both ends.

[0028] In one embodiment, the first adaptor can be ligated or appended to the 5' and/or 3' ends of the nucleic acid fragments generated by the methods described herein. The first adaptor can comprise two strands wherein each strand comprises a free 3' hydroxyl group but neither strand comprises a free 5' phosphate. In one embodiment, the free 3' hydroxyl group on each strand of the first adaptor can be ligated to a free 5' phosphate present on either end of the nucleic acid fragments of the present invention. In this

embodiment, the first adaptor comprises a ligation strand and a non-ligation strand whereby the ligation strand can be ligated to the 5' phosphate on either end of the nucleic acid fragment while a nick or gap can be present between the non-ligation strand of the first adaptor and the 3' hydroxyl on either end of the nucleic acid fragment. In one embodiment, the nick or gap can be filled in by performing a gap repair reaction. In one embodiment, the gap repair can be performed with a DNA dependent DNA polymerase with strand displacement activity. In one embodiment, the gap repair can be performed using a DNA dependent DNA polymerase with weak or no strand displacement activity. In one embodiment, the ligation strand of the first adaptor can serve as the template for the gap repair or fill-in reaction. In this embodiment, the gap repair or fill-in reaction comprises an extension reaction wherein the ligation strand of the first adaptor serves as a template and leads to the generation of nucleic acid fragments with complementary termini or ends as depicted, for example, in FIG. 1. In one embodiment, the gap repair can be performed using *Taq* DNA polymerase. In one embodiment, the ligation of the first adaptor to the nucleic acid fragments generated by the methods described herein may not be followed gap repair as depicted, for example, in FIG. 2. In this embodiment, the nucleic acid fragments comprise first adaptor sequence ligated only at the 5' end of each strand.

[0029] Ligation and, optionally gap repair, of the first adaptor to the nucleic acid fragments generates a first adaptor-nucleic acid fragment complex. In one embodiment, the first adaptor-nucleic acid fragment complex can be denatured. Denaturation can be achieved using any of the methods known in the art including, but not limited to, physical, thermal, and/ or chemical denaturation. In one embodiment, denaturation can be achieved using thermal or heat denaturation. In one embodiment, denaturation of the first adaptor-nucleic acid fragment complex generates single stranded nucleic acid fragments comprising first adaptor sequence at only the 5' end of the nucleic acid fragments as depicted, for example, in FIG. 2. In another embodiment, denaturation of the first adaptor-nucleic acid fragment complex generates single stranded nucleic acid fragments comprising first adaptor sequence at both the 5' end and 3' end of the nucleic acid fragments as depicted, for example, in FIG. 1.

[0030] In one embodiment, the nucleic acid fragments comprising first adaptor sequence appended to either the 5' end or both the 5' and 3' end can be denatured to generate single stranded nucleic acid fragments comprising first adaptor sequence appended to either the

5' end or both the 5' and 3' end. In one embodiment, the methods of the present invention described herein can be used to generate a plurality of single stranded nucleic acid fragments comprising first adaptor sequence appended to either the 5' end or both the 5' and 3' end. In one embodiment, an oligonucleotide comprising at a first end sequence complementary to a target sequence of interest present in a single stranded nucleic acid fragment and at a second end sequence from a second adaptor, wherein the second adaptor sequence is not complementary to the target nucleic acid can be annealed to the single stranded nucleic acid fragments. In one embodiment, the second adaptor sequence can be sequence from a reverse adaptor. In one embodiment, the target nucleic acid sequence of interest can be present in one or more of the single stranded nucleic acid fragments. In one embodiment, different or distinct target nucleic acid sequences of interest can be present in one or more of the single stranded nucleic acid fragments. In one embodiment, one or more oligonucleotides can comprise sequence complementary to the same sequence of interest present in one or more single stranded nucleic acid fragments. In this embodiment, the one or more oligonucleotides can comprise sequence that is complementary to different parts or regions of the same sequence of interest. In one embodiment, the different regions can be adjacent to each other. In one embodiment, the different regions can be non-adjacent to each other. In a preferred embodiment, the one or more oligonucleotides that comprise sequence complementary to the same target nucleic acid sequence of interest further comprise the same second adaptor sequence. In another embodiment, one or more oligonucleotides can comprise sequence complementary to different or distinct sequences of interest which can be present in one or more single stranded nucleic acid fragments. In a preferred embodiment, the one or more oligonucleotides that comprise sequence complementary to different or distinct target nucleic acid sequences of interest further comprise the same second adaptor sequence. In one embodiment, the sequence complementary to the target sequence of interest can be at the 3' end of the oligonucleotide and the second adaptor sequence can be at the 5' end of the oligonucleotide. In a preferred embodiment, the second adaptor sequence is non-complementary to the target nucleic acid sequence of interest. In this manner, the second adaptor sequence serves as a tail. The second adaptor sequence can be a conventional adaptor sequence. In a preferred embodiment, the second adaptor sequence can be a conventional adaptor sequence that is different than or distinct from the sequence of the first adaptor appended to the single stranded nucleic acid fragment as

described above. In one embodiment, the second adaptor sequence can be of known sequence and, thus, allow generation and/or use of sequence specific primers for amplification and/or sequencing of any polynucleotides to which the second adaptor sequence is appended or attached. In a separate embodiment, the oligonucleotide can be annealed to the nucleic acid fragments comprising the first adaptor sequence appended to either the 5' end or both the 5' and 3' end without prior denaturation. In this embodiment, annealing of the oligonucleotide can be via formation of a triple helix or triplex between the oligonucleotide and a double stranded nucleic acid fragment comprising the first adaptor sequence appended to either the 5' end or both the 5' and 3' ends of the double stranded nucleic acid fragment. In this embodiment, the double stranded nucleic acid fragment comprises a sequence of interest and can be present amongst a plurality of double stranded nucleic acid fragments comprising first adaptor sequence appended to either the 5' end or both the 5' and 3' end. Further to this embodiment, the oligonucleotide comprises sequence complementary to the sequence of interest in the double stranded nucleic acid fragment. Overall, the use of the oligonucleotide comprising sequence complementary to a target sequence of interest present in a nucleic acid fragment amongst one or more or a plurality of nucleic acid fragments allows for selective binding and subsequent enrichment of said nucleic fragment using the methods described herein.

[0031] Following annealing of the oligonucleotide as described above, a polymerase can be used to extend the oligonucleotide. In one embodiment, the polymerase can be a DNA dependent DNA polymerase. In one embodiment, the DNA dependent DNA polymerase can be any of the DNA dependent DNA polymerases as described herein and extension of the oligonucleotide can be by any of the methods known in the art. In one embodiment, an oligonucleotide comprising the second adaptor sequence, wherein the second adaptor sequence is not complementary to the target nucleic acid, and sequence complementary to a target sequence of interest present in a nucleic acid fragment comprising a first adaptor appended to one and/or both ends can be annealed to the nucleic acid fragment and extended with a polymerase to generate an oligonucleotide extension product comprising the first adaptor sequence at a first end and the second adaptor sequence at a second end. In one embodiment, the nucleic acid fragment can be present amongst a plurality of nucleic acid fragments comprising first adaptor appended to one and/or both ends. In this

embodiment, the oligonucleotide extension product can only be generated for a nucleic acid fragment that contains the target sequence of interest.

[0032] In one embodiment, the oligonucleotide extension product generated by the methods described herein can be subjected to an amplification reaction. In one embodiment, the amplification reaction can be exponential, and may be carried out at various temperature cycles or isothermal. In one embodiment, the amplification can be polymerase chain reaction. In one embodiment, the amplification reaction can be isothermal. In one embodiment, the oligonucleotide extension product comprises first adaptor sequence on one end and second adaptor sequence on the other end as generated by the methods described herein. In a preferred embodiment, the oligonucleotide extension product can be separated from the template nucleic acid fragment in order to generate a single stranded oligonucleotide extension product with first adaptor sequence on the 5' end and second adaptor sequence on the 3' end. The single stranded oligonucleotide extension product can then be amplified using a first primer comprising sequence complementary to the first adaptor and a second primer comprising sequence complementary to the second adaptor sequence. In this manner only oligonucleotide extension products comprising both the first and the second adaptor sequence will be amplified and thus enriched. In one embodiment, the first adaptor and/or the second adaptor sequence can comprise an identifier sequence. In one embodiment, the identifier sequence can be barcode sequence. In one embodiment, the barcode sequence can be the same or different for the first adaptor and the second adaptor sequence. In one embodiment, the first adaptor and/or the second adaptor sequence can comprise sequence that can be used for downstream applications such as, for example, but not limited to, sequencing. In one embodiment, the first adaptor and/or the second adaptor sequence can comprise flow cell sequences which can be used for sequencing with the sequencing method developed by Illumina and described herein.

[0033] In an alternate embodiment, the methods of the present invention can be used to generate a library of nucleic acid fragments or inserts wherein each nucleic acid fragment comprises an adaptor at one or both ends. In one embodiment, the adaptors can be present at both ends and can be distinct from each other. In one embodiment, the adaptors can be present at both ends and can comprise the same adaptor sequence. The generation of the library comprising nucleic acid inserts with distinct adaptors at both ends can involve the methods for generating oligonucleotide extension products comprising first

adaptor sequence on one end and second adaptor sequence on the other end as described above with the exception that the oligonucleotide that binds to the nucleic acid fragments and can be extended comprises random sequence. In this embodiment, the oligonucleotide comprises random sequence at the 3' portion that is hybridizable to one or more nucleic acid fragments and further comprises second adaptor sequence at the 5'-portion. Extension of the oligonucleotide along the nucleic acid fragment and the corresponding first adaptor generates a product, or products, comprising the second adaptor at one end and a sequence complementary to the first adaptor at the other end, as illustrated in Figure 4. In one embodiment, the random sequence present in the oligonucleotide can bind to and be extended on one or more nucleic acid inserts. In one embodiment, one or more oligonucleotides comprising a 3' portion comprising random sequence and a 5' portion comprising second adaptor sequence can be annealed to a library comprising nucleic acid inserts comprising a first adaptor sequence on one or both ends of each of the nucleic acid inserts. In one embodiment, the first adaptor can be the same or common to each of the nucleic acid inserts. In one embodiment, the second adaptor sequence can be the same or common to each of the one or more oligonucleotides. In one embodiment, the methods described above can be used to generate a library of nucleic acid inserts comprising wherein each of the nucleic acid inserts comprises a common first adaptor on one end and a common second adaptor sequence on a second end. In one embodiment, the first adaptor and the second adaptor sequence can be distinct from each other. In one embodiment, the first adaptor and the second adaptor sequence can comprise the same adaptor sequence. Overall, the methods of the present invention as described above can be used for the high efficiency generation of a library of nucleic acid sequences.

[0034] In yet another alternate embodiment to the methods of the invention as described above, the first adaptor can be a double stranded DNA adaptor comprising a partial duplex, wherein the two strands of the adaptor can be different lengths with a complementary region and an overhanging region at the 5' end. In this embodiment, the 5' end of the long strand of the partial duplex adaptor can comprise a unique site for a nucleic acid modifying enzyme, such as a restriction enzyme, that is absent from the short strand of the duplex adaptor. In a further embodiment, the 3' end of the short strand adaptor can be modified by a replacement of the 3' OH-group by a blocking group, for example, a dideoxynucleotide (ddCMP, ddAMP, ddTMP, or ddGMP) to prevent

polymerase extension. In this embodiment, the first adaptor comprising the partial duplex can be ligated to nucleic acid fragments generated by the methods described herein. In one embodiment, ligation of the partial duplex first adaptor can be followed by a gap repair reaction as described above. In this embodiment, ligation of the partial duplex first adaptor is not followed by a gap repair reaction. In a preferred embodiment, the partial duplex first adaptor comprises a free 5' phosphate on the short strand and a free 3' hydroxyl on the long strand. In this embodiment, ligation of the partial duplex adaptor generates double stranded nucleic acid fragments wherein both ends of the double stranded nucleic acid fragment comprise the long strand and short strand of the partial duplex first adaptor. A double stranded partial duplex first adaptor-nucleic acid fragment complex can be generated by ligation. In one embodiment, the double stranded partial duplex first adaptor-nucleic acid fragment complex can be denatured to generate a single stranded nucleic acid fragment comprising the long strand of the first adaptor on a first end and the short strand of the first adaptor on a second end. In this embodiment, the first end is the 5' end and the second end is the 3' end. In one embodiment, the first adaptor can be appended to one or more nucleic acid fragments as generated by the methods described herein such that each of the nucleic acid fragments comprises the same first adaptor or, in other words, the first adaptor can be common to each of the nucleic acid inserts. An oligonucleotide or primer comprising sequence complementary to a sequence of interest in the single stranded nucleic acid fragment can be annealed to the single stranded nucleic acid fragment and extended using a polymerase. In one embodiment, the polymerase can be a DNA dependent DNA polymerase. In one embodiment, the DNA dependent DNA polymerase can be any of the DNA dependent DNA polymerases as described herein and extension of the oligonucleotide can be by any of the methods known in the art. Extension of the primer annealed to the single stranded nucleic acid fragment generates an oligonucleotide extension product comprising sequence complementary to the long strand of the first adaptor on one end. In one embodiment, the oligonucleotide extension product remains hybridized to the single stranded nucleic acid fragment such that the restriction and/or cleavage site specific for a nucleic acid modifying enzyme is made double stranded. The double stranded site can then be cleaved by the nucleic acid modifying enzyme specific for the double stranded restriction site. In one embodiment, the nucleic acid modifying enzyme can be a restriction enzyme. In one embodiment, the restriction enzyme can be specific for a double stranded restriction site.

In one embodiment, cleavage of the restriction site can generate a blunt end or non-blunt end. In one embodiment, end repair by any of the methods described herein can be performed on the end of the nucleic acid fragment following cleavage. Cleavage of the restriction and/or cleavage site generates a site to which a second adaptor can be ligated. Ligation of the second adaptor can be through any of the methods for ligation as described herein. In one embodiment, ligation generates a double stranded nucleic acid fragment comprising the second adaptor on a first end and a partial duplex on a second end, wherein the partial duplex comprises a 3' overhang comprising the sequence of the short strand of the first adaptor. The double stranded nucleic acid fragment can then be denatured using any of the methods for denaturation disclosed herein to generate a single stranded nucleic acid fragment comprising the second adaptor sequence on the first end and the sequence of the short strand of the first adaptor on the second end. In one embodiment, the first end and second end comprise the 5' end and 3' end, respectively. In one embodiment, the second adaptor can be appended to one or more nucleic acid fragments following cleavage of the double stranded restriction site such that each of the nucleic acid fragments comprises the same second adaptor or, in other words, the second adaptor can be common to each of the nucleic acid inserts. The single stranded nucleic acid fragment can then be amplified using a first primer specific for the second primer and a second primer specific for sequence present in the short strand of the first adaptor. In one embodiment, the amplification reaction can be exponential, and may be carried out at various temperature cycles or isothermal. In one embodiment, the amplification can be polymerase chain reaction. In one embodiment, the amplification reaction can be isothermal. Overall, only a fragment comprising the second adaptor and the short strand of the first adaptor will be amplified or enriched. In so far as the method provides for enrichment of targeted fragments of the library, and not enrichment of oligonucleotide extension products generated by the extension of the oligonucleotide comprising sequence complementary to a target sequence of interest, there is no distortion of the original DNA library, and the enrichment is independent of the insert length. Because the 3' end of the short strand of the partial duplex adaptor is 3' blocked, the method enables directional or asymmetric ligation. In one embodiment, the oligonucleotide that comprises sequence complementary to a sequence of interest in a nucleic acid fragment further comprises reverse adaptor sequence. In this embodiment, the sequence complementary to a sequence of interest in the nucleic acid fragment can be present in a 3' portion of the

oligonucleotide and the reverse adaptor sequence can be present at a 5' portion. Further to this embodiment, the reverse adaptor sequence can be a common or conventional adaptor sequence and can be different or distinct from the first and/or second adaptors. Further still to this embodiment, the methods described above can lead to the generation of a single stranded nucleic acid fragment comprising the second adaptor at one end and the reverse adaptor sequence at the other end. Subsequent to this embodiment, the single stranded nucleic acid fragment can be enriched through amplification using a first primer specific to the second adaptor and a second primer specific to the third adaptor sequence.

[0035] The methods of the inventions are further applicable to any enrichment of target nucleic acid sequences of interest from libraries comprising fragments of nucleic acid of a sample appended with adaptor sequence at one or both ends, wherein the libraries are generated using ligation of the adaptor or adaptor sequences to one or both ends as described herein or by ligation independent methods, such as for example Nextera, a transposome driven method. In one embodiment, the nucleic acid can be DNA such as genomic DNA or cDNA. In one embodiment, the nucleic acid can be double stranded. Enrichment of nucleic acid sequences of interest can be achieved using the methods described herein for target enrichment. In one embodiment, the method for enriching for target nucleic acid sequences of interest from a library comprising nucleic inserts with adaptors appended to one or both ends comprises denaturing the nucleic acid inserts to generate a library of single stranded nucleic acid inserts. In one embodiment, each of the nucleic acid inserts can comprise a first adaptor sequence on one end and a second adaptor sequence on an opposite end. In one embodiment, the first adaptor and the second adaptor can be distinct from each other. In one embodiment, the first adaptor and the second adaptor can comprise the same adaptor sequence. In one embodiment, each of the nucleic acid inserts can comprise a first adaptor sequence on one end and a second adaptor sequence on an opposite end such that denaturation generates a library of single stranded nucleic acid inserts comprising the first adaptor sequence on one end and the second adaptor sequence on an opposite end. Denaturation can be achieved using any of the methods described herein. Further to the embodiments described above, one or more oligonucleotides can be annealed to the single stranded nucleic acid inserts. In one embodiment, each of the one or more oligonucleotides comprises a 3' portion that is complementary to a target nucleic acid sequence of interest present in one or more of the nucleic acid inserts, and a 5' portion comprising a third adaptor sequence. In one

embodiment, the third adaptor sequence is distinct from either or both of the first adaptor and the second adaptor. The one or more oligonucleotides can be extended with a polymerase (i.e. a DNA polymerase) thereby generating one or more oligonucleotide extension products with the first or second adaptor at a first end and the third adaptor sequence at a second end. In one embodiment, the first end comprises the 5' end and the second end comprises the 3' end. The one or more oligonucleotide extension products can be amplified using a first primer that can be complementary to the first or second adaptor and a second primer that can be complementary to the third adaptor sequence to enrich for nucleic acid fragments comprising the first or second adaptor and the third adaptor sequence at each end. In one embodiment, the first and second adaptors can be common to each of the nucleic acid inserts in the library. In one embodiment, the third adaptor sequence can be common to each of the one or more oligonucleotides. Overall, the target enrichment methods as described above can be used to generate a composition comprising a library of nucleic acid inserts enriched for any target sequence of interest from a non-enriched library comprised of nucleic acid inserts with an adaptor ligated to one or both ends.

[0036] A schematic of a preferred embodiment of the methods described herein for enriching for target sequences of interest is illustrated in FIG. 1. Overall, FIG. 1 depicts a method for isolating or enriching for a nucleic acid fragment or insert comprising a target nucleic acid sequence from a library or plurality of nucleic acid fragments. The method in FIG. 1 involves generation of a ligated library of nucleic acid fragments or inserts wherein each fragment or insert of the ligated library comprises a common forward adaptor and a fragment or insert specific reverse adaptor distinct from the forward adaptor such that subtractive PCR using a primer directed against the common forward adaptor and a primer directed against the reverse adaptor enriches for a nucleic acid fragment or insert comprising a target nucleic acid sequence. The input for the method depicted in FIG.1 is fragmented DNA. The fragmented DNA is double stranded and comprises a plurality or library of DNA fragments. In one embodiment, the DNA fragments can be derived from complex DNA, such as double-stranded DNA, genomic DNA or mixed DNA from more than one organism. In one embodiment, the DNA fragments can be derived from RNA that has been converted to cDNA through a first strand synthesis reaction using any of the methods well known in the art for generating cDNA from an RNA template which can include, but is not limited to, combining the RNA with a primer

(i.e. random primer), and reverse transcribing the RNA template with an RNA-dependent DNA polymerase. In one embodiment, the DNA fragments can be derived from RNA that has been converted to double stranded cDNA through a first and second strand synthesis reaction using any of the methods well known in the art. Fragmentation of the DNA to produce the DNA fragments can be achieved through any of the methods described herein for fragmenting nucleic acids which can include, but are not limited to, physical (i.e. sonication), and/or chemical (i.e. restriction enzyme treatment) fragmentation reactions.

[0037] As depicted in FIG. 1, a single forward adaptor is ligated to the DNA fragments. In one embodiment, the single forward adaptor can comprise known sequence. In one embodiment, the single forward adaptor can be a common adaptor. In one embodiment, the DNA fragments can be subjected to an end repair reaction as described herein to produce blunt ends. In this embodiment, the single forward adaptor can also comprise blunt ends and ligation between the single forward adaptor and the DNA fragments can be through blunt end ligation as described herein. Ligation can be facilitated through the use of enzymes (i.e. T4 DNA ligase) and methods known in the art, including, but not limited to, commercially available kits such as the EncoreTM Ultra Low Input NGS Library System. In FIG. 1, the forward adaptor can contain a strand (the ligation strand) that ligates with the free 5'phosphate on a 5' end of the DNA fragments and a strand that does not ligate (non-ligation strand) to a 3' end of the DNA fragments. In one embodiment, the ligation reaction can lead to the generation of a nick or gap between the non-ligation strand of the single forward adaptor and the 3' end of the DNA fragments. In this embodiment, the nick or gap can be repaired or filled in through a gap repair or fill-in reaction wherein the 3' end of the DNA fragments can be extended with a polymerase (preferably with a DNA dependent DNA polymerase such as *Taq* DNA polymerase) wherein the ligation strand of the forward adaptor can serve as template. In this embodiment, the gap repair generates DNA fragments with complementary ends. As depicted in FIG. 1, the DNA fragments with complementary ends are denatured to generate a denatured library comprising single stranded DNA fragments with complementary ends. Denaturation can be achieved using any of the methods known in the art which can include, but are not limited to, heat denaturation, and/or chemical denaturation.

[0038] As depicted in FIG. 1, a custom oligonucleotide with a reverse adaptor tail is annealed to the single stranded DNA fragments with complementary ends. In one embodiment, the custom oligonucleotide with a reverse adaptor tail can comprise a 3' portion comprising sequence complementary to a target sequence of interest in one of the single-stranded DNA fragments and a 5' portion comprising reverse adaptor sequence that is not complementary to the single-stranded DNA fragments in the denatured library. In one embodiment, the reverse adaptor sequence can be known sequence. In a preferred embodiment, the reverse adaptor sequence can be distinct from the single forward adaptor as described herein. In one embodiment, a plurality of custom oligonucleotides with a reverse adaptor tail can be added to the denatured library wherein the plurality of custom oligonucleotides with a reverse adaptor tail comprise a 3' portion comprising sequence complementary to a target sequence of interest in one or more of the single-stranded DNA fragments of the denatured library and a 5' portion comprising a reverse adaptor sequence that is not complementary to the single-stranded DNA fragments in the denatured library. In one embodiment, the reverse adaptor tail comprises the same reverse adaptor sequence in each of the plurality of custom oligonucleotides with a reverse adaptor tail, and wherein the reverse adaptor sequence is distinct from the forward adaptor sequence. In another embodiment, the reverse adaptor tail comprises a different reverse adaptor sequence for each of the plurality of custom oligonucleotides with a reverse adaptor tail, and wherein each of the different reverse adaptor sequences is distinct from the forward adaptor sequence. In one embodiment, the 3' portion of the custom oligonucleotide with a reverse adaptor tail can be a specific sequence, wherein the custom oligonucleotide comprises a sequence complementary to the target sequence of interest and provides a means for targeted enrichment of sequence or sequences of interest using the methods of the invention. In another embodiment the 3' portion of the custom oligonucleotide with reverse adaptor tail can be a randomly generated sequence hybridizable to random sequences of the library of fragments with adaptor sequences on one or both ends, providing means for efficient, non-enriched library generation employing the methods of the invention.

[0039] Following annealing of the custom oligonucleotide with a reverse adaptor tail to a sequence of interest in a single-stranded DNA fragment of the denatured library, the custom oligonucleotide with a reverse adaptor tail is extended using any method known in the art, which can include but is not limited to, extension using a DNA dependent DNA

polymerase using the single stranded DNA fragment of the denatured library as a template. Extension of the custom oligonucleotide with a reverse adaptor tail generates an oligonucleotide extension product with forward adaptor sequence at one end and reverse adaptor sequence at the other end. In this embodiment, the custom oligonucleotide with a reverse adaptor tail can only anneal to and be extended on DNA fragments in the denatured library comprising the target sequence of interest for which the custom oligonucleotide with a reverse adaptor tail is directed. As illustrated in FIG. 1, a subtractive polymerase chain reaction (PCR) procedure is subsequently performed using a first primer directed against the forward adaptor sequence and a second primer directed toward the reverse adaptor sequence such that only the oligonucleotide extension product with the forward adaptor sequence at one end and the reverse adaptor sequence at the other end can be amplified and thus enriched.

[0040] FIG. 2 depicts another embodiment of subtractive PCR enrichment method as described for FIG. 1, wherein ligation of a duplex forward adaptor (P1) to a double stranded nucleic acid fragment is not subjected to gap repair. The duplex forward adaptor (P1) comprises a strand (the ligation strand; P1) that ligates with the free 5' phosphate on a 5' end of the nucleic acid fragment and a strand that does not ligate (non-ligation strand; P1rc) to a 3' end of the nucleic acid fragment. In this embodiment, the ligation strand is ligated to the 5' end of both strands of a double stranded nucleic fragment whereas a gap or nick is generated between the non-ligation strand and the 3' end of both strands of the double stranded nucleic acid fragment. As depicted in FIG. 2, the ligation of the P1 adaptor to the nucleic acid fragment is followed by denaturation without a gap repair or fill-in reaction thereby generating a single stranded nucleic acid fragment with non-complementary ends. In this embodiment, the single stranded nucleic acid fragment with non-complementary ends comprises a P1 forward adaptor sequence at a 5' end and fragment specific sequence at a 3' end. In a further embodiment, the single stranded nucleic fragment with non-complementary ends can be further processed as described above and illustrated in FIG. 1 to generate a single stranded nucleic fragment with the P1 forward adaptor sequence at the 5' end and a distinct reverse adaptor sequence on the 3' end.

[0041] FIG. 4 illustrates another embodiment of the present invention for the high efficiency generation of libraries comprising nucleic acid fragments with distinct adaptors on each end. In this embodiment, the methods for generating a denatured library

comprising single stranded DNA fragments with a single forward adaptor sequence on a 5' end and a reverse adaptor sequence on a 3' end is similar to the method described for FIG. 1. However, in FIG. 4, an oligonucleotide with a reverse adaptor tail is used whereby the oligonucleotide comprises a 3' portion comprising random sequence and a reverse adaptor tail wherein the reverse adaptor tail comprises reverse adaptor sequence that is distinct from the single forward adaptor sequence. As illustrated in FIG. 4, PCR can be carried out with a first primer directed against the single forward adaptor sequence and a second primer directed toward the reverse adaptor sequence wherein both the first and second primers further comprise flow cell sequences. In this manner, single stranded DNA fragments with a single forward adaptor sequence on a 5' end and a reverse adaptor sequence on a 3' end comprise flow cell sequences that can be used to adhere the amplified single stranded DNA fragments to flow cells for subsequent sequencing by the method commercialized by Illumina, as described U.S. Pat. Nos. 5,750,341; 6,306,597; and 5,969,119.

[0042] FIG. 3 illustrates a method for enrichment of a target nucleic acid sequence, or sequences of interest, contained in a double stranded nucleic acid insert from a complex library. In one embodiment, the complex library comprises nucleic acid inserts from a genomic DNA sample. In FIG. 3, the single forward adaptor comprises a partial duplex forward adaptor comprising a long strand, A, that forms a partial duplex with a short strand, B. Strand A of the partial duplex adaptor further comprises a restriction enzyme site while strand B does not contain the restriction enzyme site. Strand B further comprises a blocking group wherein the 3' end of strand B is modified by replacement of the 3' OH group with a blocking group that can prevent polymerase extension. In one embodiment, the partial duplex forward adaptor is ligated to the double stranded nucleic acid fragments such that a double stranded insert with the partial duplex forward adaptor appended to both ends is generated. In this embodiment, the 5' end of strand B of the partial duplex adaptor can contain a free 5' phosphate which can be ligated to a free 3' OH present on one or both strands of the double stranded insert. Subsequent denaturation generates a single stranded insert comprising sequence A on a 5' end and strand B on a 3' end. A primer, C, directed against a specific sequence of interest within the single stranded insert can be annealed to the specific sequence and extended with a DNA polymerase using the single stranded insert as a template. In one embodiment, primer C can be a sequence specific primer and is employed for enrichment of target, or targets, of

interest according to the methods of the invention. In one embodiment, primer C can be a random primer. Extension of primer C with DNA polymerase generates an extended primer C product that comprises sequence complementary to sequence A at its' 3' end in a double stranded complex with the template insert strand such that a double stranded restriction site has been generated between sequence A and its' complement. In one embodiment, the double stranded restriction enzyme recognition site can be cleaved by a restriction enzyme specific for the double stranded restriction site thereby generating a truncated, or cleaved, adaptor sequence at one end. A second forward adaptor comprising a common, or conventional, duplex adaptor D is then ligated to the cleavage site using any of the ligation methods described herein, thereby generating a double stranded complex comprising the second forward adaptor D at one end and a 3' overhang comprising strand B on the opposite end. The double stranded complex comprising the second forward adaptor D at one end and a 3' overhang comprising strand B on the opposite end is denatured and amplified using a first primer directed against the second forward adaptor D and a second primer directed against strand B. In this manner, the methods depicted in FIG. 3 can be used to enrich for specific sequences of interest from a complex library since the methods are designed such that the second forward adaptor D can only bind to the double stranded cleavage site generated by restriction enzyme digestion of the double stranded restriction site created between sequence A and its complement following extension of primer C. As described, primer C can be directed against a target sequence of interest present in a single, or multiple insert, or inserts, amongst a plurality of inserts. Moreover, the method can be made strand specific by designing primer C to bind to target sequence of interest present on one strand or the other of an insert in an amongst a plurality of inserts.

[0043] Unless otherwise specified, terms and symbols of genetics, molecular biology, biochemistry and nucleic acid used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, *DNA Replication*, Second Edition (W.H. Freeman, New York, 1992); Lehninger, *Biochemistry*, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, *Human Molecular Genetics*, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, *Oligonucleotides and Analogs: A Practical Approach* (Oxford University Press, New York, 1991); Gait, editor, *Oligonucleotide Synthesis: A Practical Approach* (IRL Press, Oxford, 1984); and the like.

Input Nucleic Acid

[0044] The input can be a nucleic acid. In one embodiment, the input can be DNA. In one embodiment, the input nucleic acid can be complex DNA, such as double-stranded DNA, genomic DNA or mixed DNA from more than one organism. In one embodiment, the input can be RNA. In one embodiment, the RNA can be obtained and purified using standard techniques in the art and include RNAs in purified or unpurified form, which include, but are not limited to, mRNAs, tRNAs, snRNAs, rRNAs, retroviruses, small non-coding RNAs, microRNAs, polysomal RNAs, pre-mRNAs, intronic RNA, viral RNA, cell free RNA and fragments thereof. The non-coding RNA, or ncRNA may include snoRNAs, microRNAs, siRNAs, piRNAs and long nc RNAs. In one embodiment, the input nucleic acid can be cDNA. The cDNA can be generated from RNA, e.g., mRNA. The cDNA can be single or double stranded. The input DNA can be of a specific species, for example, human, rat, mouse, other animals, specific plants, bacteria, algae, viruses, and the like. The input complex also can be from a mixture of genomes of different species such as host-pathogen, bacterial populations and the like. The input DNA can be cDNA made from a mixture of genomes of different species. Alternatively, the input nucleic acid can be from a synthetic source. The input DNA can be mitochondrial DNA. The input DNA can be cell-free DNA. The cell-free DNA can be obtained from, e.g., a serum or plasma sample. The input DNA can comprise one or more chromosomes. For example, if the input DNA is from a human, the DNA can comprise one or more of chromosome 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X, or Y. The DNA can be from a linear or circular genome. The DNA can be plasmid DNA, cosmid DNA, bacterial artificial chromosome (BAC), or yeast artificial chromosome (YAC). The input DNA can be from more than one individual or organism. The input DNA can be double stranded or single stranded. The input DNA can be part of chromatin. The input DNA can be associated with histones.

[0045] In some embodiments, the oligonucleotides targeting the selected sequence regions of interest are designed to hybridize to single-stranded nucleic acid targets. In one embodiment, the oligonucleotides targeting the selected sequence regions of interest are designed to hybridize to single-stranded DNA targets. In the case where the input nucleic acid sample comprises genomic DNA or other double-stranded DNA, the input nucleic acid sample can be first denatured to render the target single stranded and enable hybridization of the oligonucleotides to the desired sequence regions of interest. In these

embodiments, the methods and compositions described herein can allow for region-specific enrichment and amplification of sequence regions of interest. In some embodiments, the other double-stranded DNA can be double-stranded cDNA generated by first and second strand synthesis of one or more target RNAs.

[0046] In other embodiments, the oligonucleotides targeting the selected sequence regions of interest are designed to hybridize to double-stranded nucleic acid targets, without denaturation of the double stranded nucleic acids. In other embodiments, the oligonucleotides targeting the selected sequence regions of interest are designed to hybridize to a double-stranded DNA target, without denaturation of the dsDNA. In these embodiments, the oligonucleotides targeting the selected sequence regions of interest are designed to form a triple helix (triplex) at the selected sequence regions of interest. The hybridization of the oligonucleotides to the double-stranded DNA sequence regions of interest can be carried out without prior denaturation of the double stranded nucleic acid sample. In such embodiments, the methods and compositions described herein can allow for region-specific enrichment as well as strand-specific enrichment and amplification of sequence regions of interest. This method can be useful for generation of copies of strand specific sequence regions of interest from complex nucleic acid without the need to denature the dsDNA input DNA, thus enabling enrichment and analysis of multiplicity of sequence regions of interest in the native complex nucleic acid sample. The method can find use for studies and analyses carried out in situ, enable studies and analysis of complex genomic DNA in single cells or collection of very small well defined cell population, as well as permit the analysis of complex genomic DNA without disruption of chromatin structures.

[0047] A “target nucleic acid sequence” or “target sequence” as used herein, is a polynucleotide sequence of interest, for which enrichment is desired. The target sequence may be known or not known, in terms of its actual sequence. Generally, a “template”, as used herein, is a polynucleotide that contains the target nucleic acid sequence. The terms “target sequence,” “target nucleic acid sequence,” “target nucleotide sequence,” “regions of interest,” or “sequence of interest” and, variations thereof, are used interchangeably.

Oligonucleotides of the invention

[0048] As used within the invention, the term “oligonucleotide” refers to a polynucleotide chain, typically less than 200 residues long, most typically between 15 and

100 nucleotides long, but also intended to encompass longer polynucleotide chains.

Oligonucleotides may be single-or double-stranded. As used in this invention, the term “oligonucleotide” may be used interchangeably with the terms “primer” and “adaptor”.

[0049] As used herein, the terms “hybridization”/ “hybridizing” and “annealing” are used interchangeably and refer to the pairing of complementary nucleic acids.

[0050] The term “primer”, as used herein, can refer to a nucleotide sequence, generally with a free 3' hydroxyl group, that is capable of hybridizing with a template (such as one or more target polynucleotides, one or more target DNAs, one or more target RNAs or a primer extension product) and is also capable of promoting polymerization of a polynucleotide complementary to the template. A primer can be, for example, an oligonucleotide. It can also be, for example, a sequence of the template (such as a primer extension product or a fragment of the template created following RNase [i.e. RNase H] cleavage of a template-DNA complex) that is hybridized to a sequence in the template itself (for example, as a hairpin loop), and that is capable of promoting nucleotide polymerization. Thus, a primer can be an exogenous (e.g., added) primer or an endogenous (e.g., template fragment) primer. A primer may contain a non-hybridizing sequence that constitutes a tail of the primer. A primer may still be hybridizing to a target even though its sequences are not fully complementary to the target.

[0051] The primers of the invention are generally oligonucleotides that are employed in an extension reaction by a polymerase along a polynucleotide template, such as in PCR, SPIA or cDNA synthesis, for example. The oligonucleotide primer can be a synthetic polynucleotide that is single stranded, containing a sequence at its 3'-end that is capable of hybridizing with a sequence of the target polynucleotide. Normally, the 3' region of the primer that hybridizes with the target nucleic acid has at least 80%, preferably 90%, more preferably 95%, most preferably 100%, complementarity to a sequence or primer binding site.

[0052] “Complementary”, as used herein, can refer to complementarity to all or only to a portion of a sequence. The number of nucleotides in the hybridizable sequence of a specific oligonucleotide primer should be such that stringency conditions used to hybridize the oligonucleotide primer will prevent excessive random non-specific hybridization. Usually, the number of nucleotides in the hybridizing portion of the oligonucleotide primer will be at least as great as the defined sequence on the target polynucleotide that the oligonucleotide primer hybridizes to, namely, at least 5, at least 6,

at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least about 20, and generally from about 6 to about 10 or 6 to about 12 or 12 to about 200 nucleotides, usually about 20 to about 50 nucleotides. In general, the target polynucleotide is larger than the oligonucleotide primer or primers as described previously.

[0053] In some cases, the identity of the investigated target polynucleotide sequence is known, and hybridizable sequence specific oligonucleotides or primers can be synthesized precisely according to the antisense sequence of the aforesaid target polynucleotide sequence. In some embodiments, multiple sequence-specific oligonucleotides or primers are employed to hybridize to a multiplicity of genomic regions of interest, allowing for selective enrichment of the regions of interest. In so far as the genomic regions may be very long, multiple oligonucleotides can be designed to hybridize to different sequence regions within the genomic regions of interest. In other embodiments, when the target polynucleotide sequence is unknown, the hybridizable sequence of an oligonucleotide or primer is a random sequence. Oligonucleotides or primers comprising random sequences may be referred to as “random primers”, or “random oligonucleoties,” as described herein. In one embodiment, an oligonucleotide or primer of the present invention hybridizable to a target sequence may comprise a mixture of primers or oligonucleotides designed to hybridize to a plurality (e.g. 2, 3, 4, about 6, 8, 10, 20, 40, 80, 100, 125, 150, 200, 250, 300, 400, 500, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 7000, 8000, 10,000, 20,000, 25,000 or more) of target sequences. In some cases, the plurality of target sequences may comprise a group of related sequences, random sequences, a whole transcriptome or fraction (e.g. substantial fraction) thereof, or any group of sequences such as mRNA. In some embodiments, the primers can be directed to known sequences present in the adaptors used in the invention as described herein. In this embodiment, the primers can comprise groups of primers comprising one or more primers in each group, wherein each group of primers can be directed against distinct adaptors.

[0054] Tailed primers or oligonucleotides can be employed in certain embodiments of the invention. In general, a tailed primer comprises a 3' portion that is hybridizable to one or more target polynucleotides, and a 5' portion that is not hybridizable to the one or more target polynucleotides. In general, the non-hybridizable 5' portion does not hybridize to the one or more target polynucleotides under conditions in which the hybridizable 3'

portion of the tailed primer hybridizes to the one or more target polynucleotides. In some embodiments, the non-hybridizable 5' portion comprises an adaptor sequence. In some embodiments, the non-hybridizable 5' portion comprises a common or conventional adaptor sequence. In some embodiments, the non-hybridizable 5' portion comprises a common or conventional adaptor sequence that is distinct or different from the sequence of other adaptors used in the present invention. In some embodiments, the non-hybridizable 5' portion comprises a promoter-specific sequence. Generally, a promoter-specific sequence comprises a single-stranded DNA sequence region which, in double-stranded form is capable of mediating RNA transcription. Examples of promoter-specific sequences are known in the art, and include, without limitation, T7, T3, or SP6 RNA polymerase promoter sequences. When the tailed primer is extended with a DNA polymerase, a primer extension product with a 5' portion comprising a defined sequence can be created. This primer extension product can then have a second primer anneal to it, which can be extended with a DNA polymerase to create a double stranded product comprising a defined sequence at one end. In some embodiments, where the non-hybridizable 5' portion of one or more tailed primers comprises a promoter-specific sequence, creation of a double-stranded product comprising a defined sequence at one end generates a double-stranded promoter sequence that is capable of mediating RNA transcription. In some embodiments, a double-stranded promoter sequence can be generated by hybridizing to the promoter-specific sequence an oligonucleotide comprising a sequence complementary to the promoter-specific sequence. In some embodiments, formation of a double-stranded promoter can be followed by the generation of single-stranded RNA by RNA transcription of sequence downstream of the double-stranded promoter, generally in a reaction mixture comprising all necessary components, including but not limited to ribonucleoside triphosphates (rNTPs) and a DNA-dependent RNA polymerase. Tailed primers can comprise DNA, RNA, or both DNA and RNA. In some embodiments, the tailed primer consists of DNA.

[0055] Composite primers can be employed in certain embodiments of the invention. Composite primers are primers that are composed of RNA and DNA portions. In some aspects, the composite primer can be a tailed composite primer comprising, for example, a 3'-DNA portion and a 5'-RNA portion. In the tailed composite primer, a 3'-portion, all or a portion of which comprises DNA, is complementary to a polynucleotide; and a 5'-portion, all or a portion of which comprises RNA, is not complementary to the

polynucleotide and does not hybridize to the polynucleotide under conditions in which the 3'-portion of the tailed composite primer hybridizes to the polynucleotide target. When the tailed composite primer is extended with a DNA polymerase, a primer extension product with a 5'-RNA portion comprising a defined sequence can be created. This primer extension product can then have a second primer anneal to it, which can be extended with a DNA polymerase to create a double stranded product with an RNA/DNA heteroduplex comprising a defined sequence at one end. The RNA portion can be selectively cleaved from the partial heteroduplex to create a double-stranded DNA with a 3'-single-stranded overhang which can be useful for various aspects of the present invention including allowing for isothermal amplification using a composite amplification primer.

[0056] A "random primer," as used herein, can be a primer that generally comprises a sequence that is designed not necessarily based on a particular or specific sequence in a sample, but rather is based on a statistical expectation (or an empirical observation) that the sequence of the random primer is hybridizable (under a given set of conditions) to one or more sequences in the sample. A random primer will generally be an oligonucleotide or a population of oligonucleotides comprising a random sequence(s) in which the nucleotides at a given position on the oligonucleotide can be any of the four nucleotides, or any of a selected group of the four nucleotides (for example only three of the four nucleotides, or only two of the four nucleotides). In some cases all of the positions of the oligonucleotide or population of oligonucleotides can be any of two or more nucleotides. In other cases, only a portion of the oligonucleotide, for instance a particular region, will comprise positions which can be any of two or more bases. In some cases, the portion of the oligonucleotide which comprises positions which can be any of two or more bases is about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or about 15-20 nucleotides in length. In some cases, a random primer may comprise a tailed primer having a 3'-region that comprises a random sequence and a 5'-region that is a non-hybridizing sequence that comprises a specific, non-random sequence. The 3'-region may also comprise a random sequence in combination with a region that comprises poly-T sequences. The sequence of a random primer (or its complement) may or may not be naturally-occurring, or may or may not be present in a pool of sequences in a sample of interest. As is well understood in the art, a "random primer" can also refer to a primer that is a member of a population of primers (a plurality of random primers) which collectively are designed to hybridize to a desired

and/or a significant number of target sequences. A random primer may hybridize at a plurality of sites on a nucleic acid sequence. The use of random primers provides a method for generating primer extension products complementary to a target polynucleotide or target nucleic sequence which does not require prior knowledge of the exact sequence of the target. In some embodiments one portion of a primer is random, and another portion of the primer comprises a defined sequence. For example, in some embodiments, a 3'-portion of the primer will comprise a random sequence, while the 5'-portion of the primer comprises a defined sequence. In some embodiments a 3'-random portion of the primer will comprise DNA, and a 5'-defined portion of the primer will comprise RNA, in other embodiments, both the 3' and 5'-portions will comprise DNA. In some embodiments, the 5'-portion will contain a defined sequence and the 3'-portion will comprise a poly-dT sequence that is hybridizable to a multiplicity of RNAs in a sample (such as all mRNA). In some embodiments, a "random primer," or primer comprising a randomly generated sequence, comprises a collection of primers comprising one or more nucleotides selected at random from two or more different nucleotides, such that all possible sequence combinations of the nucleotides selected at random may be represented in the collection. In some embodiments, generation of one or more random primers does not include a step of excluding or selecting certain sequences or nucleotide combinations from the possible sequence combinations in the random portion of the one or more random primers.

[0057] In one embodiment, the oligonucleotides of the invention can be tailed oligonucleotides. In one embodiment, the 5'-tail can comprise RNA and is non hybridizable to the RNA in the sample. In one embodiment, the 5'-tail can comprise DNA and is non hybridizable to the DNA in the sample. In one embodiment, the 5'-tail can comprise an adaptor that is not hybridizable to the DNA and/or nucleic acid fragments derived from the sample comprising nucleic acid. In one embodiment, the 5'-tail can comprise an adaptor sequence that is not hybridizable to the DNA and/or nucleic acid fragments derived from the sample comprising nucleic acid. In some embodiments, the 5'-tail can comprise a common adaptor sequence that is not hybridizable to the DNA and is distinct from any other adaptor or adaptor sequence used in the methods of the invention described herein. In some embodiments, the 5'-tail can comprise an identifier sequence. In some embodiments, the identifier sequence can comprise a barcode

sequence. In some embodiments, the 5'-tail can comprise a common adaptor sequence that is not hybridizable to the DNA and a barcode sequence.

[0058] The term "adaptor", as used herein, refers to an oligonucleotide of known sequence, the ligation of which to a target polynucleotide or a target polynucleotide strand of interest enables the generation of amplification-ready products of the target polynucleotide or the target polynucleotide strand of interest. The target polynucleotide molecules may be fragmented or not prior to the addition of adaptors.

[0059] Various adaptor designs are envisioned which are suitable for generation of amplification-ready products of target sequence regions/strands of interest. For example, the two strands of the adaptor may be self-complementary, non-complementary or partially complementary. A common feature of the adaptors depicted in Figure 3 of the invention is the partial duplex design, wherein the two strands of the adaptor are different lengths with a complementary region and an overhanging region at the 5' end. The 5' end of the long strand of the partial duplex adaptor contains a unique site for a nucleic acid modifying enzyme, such as a restriction enzyme, that is absent from the short strand of the duplex adaptor. The 3' end of the short strand adaptor is modified by a replacement of the 3' OH-group by a blocking group, for example, a dideoxynucleotide (ddCMP, ddAMP, ddTMP, or ddGMP) to prevent polymerase extension.

[0060] In some embodiments of the invention, the adaptors comprise an additional identifier sequence, i.e. a barcode sequence. As used herein, the term "barcode" refers to a known nucleic acid sequence that allows some feature of a polynucleotide with which the barcode is associated to be identified. In some embodiments, the feature of the polynucleotide to be identified is the sample from which the polynucleotide is derived. In some embodiments, barcodes are at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more nucleotides in length. In some embodiments, barcodes are shorter than 10, 9, 8, 7, 6, 5, or 4 nucleotides in length. In some embodiments, each barcode in a plurality of barcodes differ from every other barcode in the plurality at at least three nucleotide positions, such as at least 3, 4, 5, 6, 7, 8, 9, 10, or more positions. In some embodiments, barcodes associated with some polynucleotides are of different length than barcodes associated with other polynucleotides. In general, barcodes are of sufficient length and comprise sequences that are sufficiently different to allow the identification of samples based on barcodes with which they are associated. In some embodiments, both the forward and reverse adapter comprise at least one of a plurality of barcode sequences. In some

embodiments, the first, second, and/or third adaptor comprises at least one of a plurality of barcode sequences. In some embodiments, each reverse adapter comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in the plurality of barcode sequences. In some embodiments, both the first adapter and the second adapter comprise at least one of a plurality of barcode sequences. In some embodiments, barcodes for second adapter oligonucleotides are selected independently from barcodes for first adapter oligonucleotides. In some embodiments, first adapter oligonucleotides and second adapter oligonucleotides having barcodes are paired, such that adapters of the pair comprise the same or different one or more barcodes. In some embodiments, the methods of the invention further comprise identifying the sample from which a target polynucleotide is derived based on the barcode sequence to which the target polynucleotide is joined. In general, a barcode comprises a nucleic acid sequence that when joined to a target polynucleotide serves as an identifier of the sample from which the target polynucleotide was derived.

[0061] Recently, many improvements have been made in adaptor design that have reduced the occurrence of adapter dimer. These improvements include the use of nucleotide analogs and structured oligonucleotides, and have allowed for use of higher concentrations of oligonucleotides in ligation reactions. The higher concentrations of adapters in ligation reactions have enabled researchers to produce high quality libraries from as few as 150 copies of genome. Ligation of adaptors to the ends of DNA fragments, in particular those fragments containing the regions of interest is suitable for carrying out the methods of the invention. Various ligation modalities are envisioned, dependent on the choice of nucleic acid modifying enzymes and the resulting double-stranded DNA cleavage. For example, when a blunt end product comprising the target region/sequence of interest is generated, blunt end ligation can be suitable. Alternatively, where the cleavage is carried out using a restriction enzyme of known sequence specificity, leading to the generation of cleavage sites with known sequence overhangs, suitable ends of the adaptors can be designed to enable hybridization of the adaptor to the cleavage site of the sequence region of interest and subsequent ligation. Reagents and methods for efficient and rapid ligation of adaptors are commercially available and are known in the art.

Nucleic acid modifying enzymes

[0062] The nucleic acid (NA) -modifying enzyme can be DNA-specific modifying enzyme. The NA-modifying enzyme can be selected for specificity for double-stranded DNA. The enzyme can be a duplex-specific endonuclease, a blunt-end frequent cutter restriction enzyme, or other restriction enzyme. Examples of blunt-end cutters include DraI or SmaI. The NA-modifying enzyme can be an enzyme provided by New England Biolabs . The NA-modifying enzyme can be a homing endonuclease (a homing endonuclease can be an endonuclease that does not have a stringently-defined recognition sequence). The NA-modifying enzyme can be a nicking endonuclease (a nicking endonuclease can be an endonuclease that can cleave only one strand of DNA in a double-stranded DNA substrate). The NA-modifying enzyme can be a high fidelity endonuclease (a high fidelity endonuclease can be an engineered endonuclease that has less “star activity” than the wild-type version of the endonuclease).

[0063] In a preferred embodiment, the NA-modifying enzyme is a sequence and duplex-specific, DNA modifying enzyme.

DNA-Dependent DNA Polymerases

[0064] DNA-dependent DNA polymerases for use in the methods and compositions of the invention are capable of effecting extension of a primer or oligonucleotide according to the methods of the invention. In one embodiment, a preferred DNA-dependent DNA polymerase can be one that is capable of extending a nucleic acid primer in the presence of the DNA and/or cDNA template. Exemplary DNA dependent DNA polymerases suitable for the methods of the present invention include but are not limited to Klenow polymerase, with or without 3'-exonuclease, Bst DNA polymerase, Bca polymerase, .phi.29 DNA polymerase, Vent polymerase, Deep Vent polymerase, Taq polymerase, T4 polymerase, and E. coli DNA polymerase 1, derivatives thereof, or mixture of polymerases. In some cases, the polymerase does not comprise a 5'-exonuclease activity. In other cases, the polymerase comprises 5' exonuclease activity. In some cases, the primer or oligonucleotide extension of the present invention may be performed using a polymerase comprising strong strand displacement activity such as for example Bst polymerase. In other cases, the primer extension of the present invention may be performed using a polymerase comprising weak or no strand displacement activity. One skilled in the art may recognize the advantages and disadvantages of the use of strand

displacement activity during the primer extension step, and which polymerases may be expected to provide strand displacement activity (see e.g., New England Biolabs Polymerases).

Methods of Amplification

[0065] The methods, compositions and kits described herein can be useful to generate amplification-ready products for downstream applications such as massively parallel sequencing (i.e. next generation sequencing methods), generation of libraries with enriched population of sequence regions of interest, or hybridization platforms. Methods of amplification are well known in the art. Suitable amplification reactions can be exponential or isothermal and can include any DNA amplification reaction, including but not limited to polymerase chain reaction (PCR), strand displacement amplification (SDA), linear amplification, multiple displacement amplification (MDA), rolling circle amplification (RCA), single primer isothermal amplification (SPIA, see e.g. U.S. Pat. No. 6,251,639), Ribo-SPIA, or a combination thereof. In some cases, the amplification methods for providing the template nucleic acid may be performed under limiting conditions such that only a few rounds of amplification (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 etc.), such as for example as is commonly done for cDNA generation. The number of rounds of amplification can be about 1-30, 1-20, 1-15, 1-10, 5-30, 10-30, 15-30, 20-30, 10-30, 15-30, 20-30, or 25-30.

[0066] PCR is an in vitro amplification procedure based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by thermophilic template dependent polynucleotide polymerase, resulting in the exponential increase in copies of the desired sequence of the polynucleotide analyte flanked by the primers. The two different PCR primers, which anneal to opposite strands of the DNA, are positioned so that the polymerase catalyzed extension product of one primer can serve as a template strand for the other, leading to the accumulation of a discrete double stranded fragment whose length is defined by the distance between the 5' ends of the oligonucleotide primers.

[0067] LCR uses a ligase enzyme to join pairs of preformed nucleic acid probes. The probes hybridize with each complementary strand of the nucleic acid analyte, if present,

and ligase is employed to bind each pair of probes together resulting in two templates that can serve in the next cycle to reiterate the particular nucleic acid sequence.

[0068] SDA (Westin et al 2000, Nature Biotechnology, 18, 199-202; Walker et al 1992, Nucleic Acids Research, 20, 7, 1691-1696), is an isothermal amplification technique based upon the ability of a restriction endonuclease such as HincII or BsoBI to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of an exonuclease deficient DNA polymerase such as Klenow exo minus polymerase, or Bst polymerase, to extend the 3'-end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions in which strands displaced from a sense reaction serve as targets for an antisense reaction and vice versa.

[0069] Some aspects of the invention utilize linear amplification of nucleic acids or polynucleotides. Linear amplification generally refers to a method that involves the formation of one or more copies of the complement of only one strand of a nucleic acid or polynucleotide molecule, usually a nucleic acid or polynucleotide analyte. Thus, the primary difference between linear amplification and exponential amplification is that in the latter process, the product serves as substrate for the formation of more product, whereas in the former process the starting sequence is the substrate for the formation of product but the product of the reaction, i.e. the replication of the starting template, is not a substrate for generation of products. In linear amplification the amount of product formed increases as a linear function of time as opposed to exponential amplification where the amount of product formed is an exponential function of time.

[0070] In some embodiments, the amplification is exponential, e.g. in the enzymatic amplification of specific double stranded sequences of DNA by a polymerase chain reaction (PCR). In other embodiments the amplification method is linear. In other embodiments the amplification method is isothermal.

Downstream Applications

[0071] An important aspect of the invention is that the methods and compositions disclosed herein can be efficiently and cost-effectively utilized for downstream analyses, such as next generation sequencing or hybridization platforms, with minimal loss of biological material of interest. The methods of the present invention can also be used in the analysis of genetic information of selective genomic regions of interest (e.g., analysis

of SNPs or other disease markers) as well as genomic regions which may interact with the selective region of interest.

Sequencing

[0072] For example, the methods of the invention are useful for sequencing by the method commercialized by Illumina, as described U.S. Pat. Nos. 5,750,341; 6,306,597; and 5,969,119. In general, double stranded fragment polynucleotides can be prepared by the methods of the present invention to produce amplified nucleic acid sequences tagged at one (e.g., (A)/(A') or both ends (e.g., (A)/(A') and (C)/(C')). In some cases, single stranded nucleic acid tagged at one or both ends is amplified by the methods of the present invention (e.g., by SPIA or linear PCR). The resulting nucleic acid is then denatured and the single-stranded amplified polynucleotides are randomly attached to the inside surface of flow-cell channels. Unlabeled nucleotides are added to initiate solid-phase bridge amplification to produce dense clusters of double-stranded DNA. To initiate the first base sequencing cycle, four labeled reversible terminators, primers, and DNA polymerase are added. After laser excitation, fluorescence from each cluster on the flow cell is imaged. The identity of the first base for each cluster is then recorded. Cycles of sequencing are performed to determine the fragment sequence one base at a time.

[0073] In some embodiments, the methods of the invention are useful for preparing target polynucleotides for sequencing by the sequencing by ligation methods commercialized by Applied Biosystems (e.g., SOLiD sequencing). In other embodiments, the methods are useful for preparing target polynucleotides for sequencing by synthesis using the methods commercialized by 454/Roche Life Sciences, including but not limited to the methods and apparatus described in Margulies et al., *Nature*(2005) 437:376-380 (2005); and U.S. Pat. Nos. 7,244,559; 7,335,762; 7,211,390; 7,244,567; 7,264,929; and 7,323,305. In other embodiments, the methods are useful for preparing target polynucleotide(s) for sequencing by the methods commercialized by Helicos BioSciences Corporation (Cambridge, Mass.) as described in U.S. application Ser. No. 11/167,046, and U.S. Pat. Nos. 7,501,245; 7,491,498; 7,276,720; and in U.S. Patent Application Publication Nos. US20090061439; US20080087826; US20060286566; US20060024711; US20060024678; US20080213770; and US20080103058. In other embodiments, the methods are useful for preparing target polynucleotide(s) for sequencing by the methods commercialized by Pacific Biosciences as described in U.S. Pat. Nos. 7,462,452;

7,476,504; 7,405,281; 7,170,050; 7,462,468; 7,476,503; 7,315,019; 7,302,146; 7,313,308; and US Application Publication Nos. US20090029385; US20090068655; US20090024331; and US20080206764.

[0074] Another example of a sequencing technique that can be used in the methods of the provided invention is nanopore sequencing (see e.g. Soni G V and Meller A. (2007) *Clin Chem* 53: 1996-2001). A nanopore can be a small hole of the order of 1 nanometer in diameter. Immersion of a nanopore in a conducting fluid and application of a potential across it can result in a slight electrical current due to conduction of ions through the nanopore. The amount of current that flows is sensitive to the size of the nanopore. As a DNA molecule passes through a nanopore, each nucleotide on the DNA molecule obstructs the nanopore to a different degree. Thus, the change in the current passing through the nanopore as the DNA molecule passes through the nanopore can represent a reading of the DNA sequence.

[0075] Another example of a sequencing technique that can be used in the methods of the provided invention is semiconductor sequencing provided by Ion Torrent (e.g., using the Ion Personal Genome Machine (PGM)). Ion Torrent technology can use a semiconductor chip with multiple layers, e.g., a layer with micro-machined wells, an ion-sensitive layer, and an ion sensor layer. Nucleic acids can be introduced into the wells, e.g., a clonal population of single nucleic acid can be attached to a single bead, and the bead can be introduced into a well. To initiate sequencing of the nucleic acids on the beads, one type of deoxyribonucleotide (e.g., dATP, dCTP, dGTP, or dTTP) can be introduced into the wells. When one or more nucleotides are incorporated by DNA polymerase, protons (hydrogen ions) are released in the well, which can be detected by the ion sensor. The semiconductor chip can then be washed and the process can be repeated with a different deoxyribonucleotide. A plurality of nucleic acids can be sequenced in the wells of a semiconductor chip. The semiconductor chip can comprise chemical-sensitive field effect transistor (chemFET) arrays to sequence DNA (for example, as described in U.S. Patent Application Publication No. 20090026082). Incorporation of one or more triphosphates into a new nucleic acid strand at the 3' end of the sequencing primer can be detected by a change in current by a chemFET. An array can have multiple chemFET sensors.

Genetic Analysis

[0076] The methods of the present invention can be used in the analysis of genetic information of selective genomic regions of interest as well as genomic regions which may interact with the selective region of interest. Amplification methods as disclosed herein can be used in the devices, kits, and methods known to the art for genetic analysis, such as, but not limited to those found in U.S. Pat. Nos. 6,449,562, 6,287,766, 7,361,468, 7,414,117, 6,225,109, and 6,110,709. In some cases, amplification methods of the present invention can be used to amplify target nucleic acid of interest for DNA hybridization studies to determine the presence or absence of polymorphisms. The polymorphisms, or alleles, can be associated with diseases or conditions such as genetic disease. In other cases the polymorphisms can be associated with susceptibility to diseases or conditions, for example, polymorphisms associated with addiction, degenerative and age related conditions, cancer, and the like. In other cases, the polymorphisms can be associated with beneficial traits such as increased coronary health, or resistance to diseases such as HIV or malaria, or resistance to degenerative diseases such as osteoporosis, Alzheimer's or dementia.

Kits

[0077] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, the kit, in a suitable container, comprises: an adaptor or several adaptors, one or more of oligonucleotide primers and reagents for ligation, primer extension and amplification. The kit may also comprise means for purification, such as a bead suspension.

[0078] The containers of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other containers, into which a component may be placed, and preferably, suitably aliquotted. Where there is more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a container.

[0079] When the components of the kit are provided in one or more liquid solutions, the liquid solution can be an aqueous solution. However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent.

[0080] A kit will preferably include instructions for employing, the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented.

[0081] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. In some embodiments, a kit comprises a composition of the invention, in one or more containers. In some embodiments, the invention provides kits comprising adapters, primers, and/or other oligonucleotides described herein. In some embodiments, the kit further comprises one or more of: (a) a DNA ligase, (b) a DNA-dependent DNA polymerase, (c) an RNA-dependent DNA polymerase, (d) a forward adapter (e) one or more oligonucleotides comprising reverse adaptor sequence and (f) one or more buffers suitable for one or more of the elements contained in said kit. The adapters, primers, other oligonucleotides, and reagents can be, without limitation, any of those described above. Elements of the kit can further be provided, without limitation, in any of the amounts and/or combinations (such as in the same kit or same container) described above. The kits may further comprise additional agents, such as those described above, for use according to the methods of the invention. For example, the kit can comprise a first forward adaptor that is a partial duplex adaptor as described herein, a second forward adapter, and a nucleic acid modifying enzyme specific for a restriction and/or cleavage site present in the first forward adaptor. The kit elements can be provided in any suitable container, including but not limited to test tubes, vials, flasks, bottles, ampules, syringes, or the like. The agents can be provided in a form that may be directly used in the methods of the invention, or in a form that requires preparation prior to use, such as in the reconstitution of lyophilized agents. Agents may be provided in aliquots for single-use or as stocks from which multiple uses, such as in a number of reaction, may be obtained.

[0082] In one embodiment, the kit comprises a plurality of forward adaptor oligonucleotides, wherein each of said forward adaptor oligonucleotides comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions, and instructions for using the same. Forward adapters comprising different barcode sequences can be supplied individually or in combination with one or more additional forward adapters having a different barcode sequence. In some embodiments, the kit can comprises a plurality of

first and second forward adapter oligonucleotides. Second forward adapter oligonucleotides can be supplied separately from or in combination with one or more first forward adapters, and/or one or more different second adapters. Combinations of first and second forward adapters can be supplied in accordance with combinations described above. In some embodiments, the kit can comprise a plurality of oligonucleotides comprising reverse adaptor sequence. In one embodiment, the kit can comprise a plurality of oligonucleotides comprising reverse adaptor sequence, wherein each of the plurality of oligonucleotides comprising reverse adaptor sequence further comprises sequence complementary to a specific target sequence of interest present in a nucleic acid. In one embodiment, the kit can comprise a plurality of oligonucleotides comprising reverse adaptor sequence, wherein each of the plurality of oligonucleotides comprising reverse adaptor sequence further comprises random sequence. In one embodiment, the kit comprises a plurality of oligonucleotides with reverse adaptor sequence, wherein each of said oligonucleotides with reverse adaptor sequence comprises at least one of a plurality of barcode sequences, wherein each barcode sequence of the plurality of barcode sequences differs from every other barcode sequence in said plurality of barcode sequences at at least three nucleotide positions, and instructions for using the same. Oligonucleotides with reverse adaptor sequence comprising different barcode sequences can be supplied individually or in combination with one or more additional oligonucleotides with reverse adaptor sequence having a different barcode sequence.

Products based on the Methods of the Invention

[0083] Products based on the methods of the invention may be commercialized by the Applicants under the trade name Ovation[®]. Ovation[®] is a trademark of NuGEN Technologies, Inc.

EXAMPLES

Example 1 – Characterization of the Human Oral Microbiome by Selective Enrichment of Bacterial 16S Ribosomal DNA Sequences.

[0084] Sample nucleic acid

Microbial genomic DNA is isolated from human saliva using the OMNIgene-DISCOVER sample collection kit (DNA Genotek) according to the manufacturer's instructions. Extracted DNA is then fragmented via sonication to an average length of 400 bp and purified using Agencourt AMPure XP beads (Beckman Coulter Genomics).

[0085] Generation of control and test libraries with ligated forward adaptors

The NuGEN Ovation Ultralow Library System (NuGEN Technologies) is used to generate two next generation sequencing libraries from 100 ng of the purified sample. The first library, an unenriched control, is made as recommended by the manufacturer. A second 'test' library, the input for downstream enrichment steps, is generated using the same library construction kit modified as follows. Briefly, DNA is blunted and prepared for ligation under the standard end-repair reaction conditions described in the kit. Fragments are then ligated to the forward adaptor only. As depicted in Figure 2, ligation attaches the forward adaptor to each end of each DNA fragment, leaving a single-strand nick on the opposite strand. Adaptor fill-in will be performed, thus generating ligation products where complementary ends exist on each insert.

[0086] Ligation products of at least 100 bp in length are purified by selective binding to Agencourt AMPure XP beads and taken forward into the enrichment process.

[0087] Amplification

Ribosomal DNA fragments from the test library are selectively amplified with two distinct steps: 1) gene-specific primer extension; and 2) PCR with universal adaptor sequences. The primer extension step is performed with oligonucleotides containing a 3' gene-specific region and a 5' common region that contains a portion of the Illumina reverse adaptor sequence. Consensus 16S sequences making up the gene-specific segment are selected by comparing the ribosomal operons from 40 diverse bacterial species using the ClustalW multiple sequence alignment program (European Bioinformatics Institute). Oligonucleotides representing each of the 18 highly conserved sequence blocks identified across the 16S genomic loci are synthesized and mixed in equimolar proportions.

[0088] The pool of primer extension probes is combined with the test DNA library (above) containing the forward adaptor and the HotStarTaq PCR mastermix (QIAGEN, USA) containing buffer, dNTPs, and a thermally-activated *Taq* DNA polymerase. This solution is placed in a thermal cycler, heated to 95°C for 15 minutes to activate the polymerase and cooled to 70°C for 5 minutes to allow the 16S primers to anneal to DNA inserts and extend into the forward adaptor site. Amplification primers that bind to the forward and reverse adaptor sites are added. Selection for fragments that contain both the forward (test library) and reverse (5' common region on 16S primers) adaptor, and the respective universal priming sites, is accomplished with PCR using a 3-step temperature routine (94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute) for 25 cycles.

PCR products are purified using AMPure XP beads and analyzed with a 2100 Bioanalyzer (Agilent Technologies).

[0089] Sequencing and data analysis

[0090] Single end sequencing reads of 100 nt length are obtained for both the control and enriched test libraries using a MiSeq System (Illumina). Raw sequencing data is processed using Illumina base calling software and mapped to a ribosomal RNA database. Sequences that do not align to bacterial rRNA are mapped to human and bacterial full genome reference sequences. Fold enrichment is determined by calculating the number of rRNA reads as a percentage of total mapped reads in the control and test samples.

Example 2 – Characterization of changes over time to the Human Oral Microbiome by Selective Enrichment of Bacterial 16S Ribosomal DNA Sequences.

[0091] Sample nucleic acid

Microbial genomic DNA is isolated from human saliva using the OMNIgene-DISCOVER sample collection kit (DNA Genotek) according to the manufacturer's instructions at 1 hour intervals for 16 hours following use of dental rinse. Extracted DNA is then fragmented via sonication to an average length of 400 bp and purified using Agencourt AMPure XP beads (Beckman Coulter Genomics).

[0092] Generation of DNA fragments with ligated forward adapters

Components from the NuGEN Ovation Ultralow Library System (NuGEN Technologies) are used to generate 16 independent next generation sequencing libraries from 100 ng of the purified sample. Briefly, DNA is blunted and prepared for ligation under the standard end-repair reaction conditions described in the kit. Fragments are then ligated to the forward adapter only. As depicted in Figure 2, ligation attaches the forward adapter to each end of each DNA fragment, leaving a single-strand nick on the opposite strand. Adapter fill-in will be performed, thus generating ligation products where complementary ends exist on each insert.

[0093] Ligation products of at least 100 bp in length are purified by selective binding to Agencourt AMPure XP beads and taken forward into the enrichment process.

[0094] Primer extension

Libraries containing ribosomal genes are generated by introducing the reverse adapter attached to the 5' end of oligonucleotides specific to conserved regions within these genes. There are two distinct steps: 1) annealing of the gene-specific primer; and 2) extension of that primer through the action of a DNA polymerase. The resulting product

is a functional library containing the forward adapter on one end and the reverse adapter on the other end. The gene-specific primer extension step is performed with oligonucleotides containing a 3' gene-specific region and a 5' region that contains a portion of the Illumina reverse adapter sequence. Embedded in the reverse adapter sequence is a variable region of 8 bases that differentiates this adapter from the 16 other adapters used with the other samples. Thus, 16 gene-specific libraries have been generated; one from each sample. Each library has a common forward adapter. Each library also contains a common sequence on the opposite end but within that common sequence there is a unique 8 nucleotide region. Consensus 16S sequences making up the gene-specific segment are selected by comparing the ribosomal operons from 40 diverse bacterial species using the ClustalW multiple sequence alignment program (European Bioinformatics Institute). Oligonucleotides representing each of the 18 highly conserved sequence blocks identified across the 16S genomic loci are synthesized and mixed in equimolar proportions.

[0095] Individual samples with forward adapters ligated onto each strand are combined with the primer extension probes (described above) in 16 independent reactions. These are mixed with HotStarTaq PCR mastermix (QIAGEN, USA) containing buffer, dNTPs, and a thermally-activatable *Taq* DNA polymerase. This solution is placed in a thermal cycler, heated to 95°C for 15 minutes to activate the polymerase and cooled to 70°C for 5 minutes to allow the 16S primers to anneal to DNA inserts and extend into the forward adapter site.

[0096] Amplification

The 16 individual primer extension products (above) are pooled, amplification primers that are complementary to the 5' ends of the forward and reverse adapter sites but also contain portions complementary to flow cell oligonucleotide sequences are added. Selection for fragments that contain both the forward and reverse (5' common region on 16S primers) adapter, and the respective universal priming sites, is accomplished with PCR using a 3-step temperature routine (94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute) for 25 cycles. PCR products are purified using AMPure XP beads and analyzed with a 2100 Bioanalyzer (Agilent Technologies).

[0097] Sequencing and data analysis

[0098] Single end sequencing reads of 100 nt length are obtained for both the control and enriched test libraries using a MiSeq System (Illumina). Raw sequencing data is

processed using Illumina base calling software. Samples from the various time points are binned based on their unique 8 base code and mapped to a ribosomal RNA database. Sequences that do not align to bacterial rRNA are mapped to human and bacterial full genome reference sequences. Changes in microbial populations are assessed by comparing 16S read counts from the different organisms in the samples over time.

Example 3 – Characterization of transcriptional activity of individual cells within a population.

[0099] Sample nucleic acid

Individual cells are isolated from whole blood using a FACS cell sorter. The cells are suspended in 10 µl of Prelude Lysis solution (a component of NuGEN Technologies, One Direct system), resulting in lysis of the cell membrane while the nuclear membrane remains intact. Sixteen of the single cell suspensions are selected for expression profiling. Briefly, kit reagents are used as described by the manufacturer to generate first and second strand cDNA from the total RNA present in the lysate. Double stranded cDNA products are purified using Agencourt AMPure XP beads (Beckman Coulter Genomics).

[00100] Generation of fragments with ligated forward adapters

Components from the NuGEN Ovation Ultralow Library System (NuGEN Technologies) are used to generate next generation sequencing libraries from each of the purified sample. Briefly, DNA is blunted and prepared for ligation under the standard end-repair reaction conditions described in the kit. Fragments are then ligated to the forward adapter only. As depicted in Figure 2, ligation attaches the forward adapter to each end of each DNA fragment, leaving a single-strand nick on the opposite strand. Adapter fill-in will be performed, thus generating ligation products where complementary ends exist on each insert.

[00101] Ligation products of at least 100 bp in length are purified by selective binding to Agencourt AMPure XP beads and taken forward into library generation.

[00102] Primer extension

Libraries are generated by introducing the reverse adapter attached to the 5' end of a random hexamer. There are two distinct steps: 1) annealing of the primer; and 2) extension of that primer through the action of a DNA polymerase. The resulting product is a functional library containing the forward adapter on one end and reverse adapter on the other end. The primer extension step is performed with oligonucleotides containing a

3' random region and a 5' region that contains a portion of the Illumina reverse adapter sequence. Embedded in the reverse adapter sequence is a variable region of 8 bases that differentiates this adapter from the 16 other adapters used with the other samples. Thus, 16 libraries have been generated; one from each sample. Each library has a common forward adapter. Each library also contains a common sequence on the opposite end but within that common sequence there is a unique 8 nucleotide region.

[00103] Individual samples with forward adapters ligated onto each strand are combined with the primer extension probes (described above) in 16 independent reactions. These are mixed with HotStarTaq PCR mastermix (QIAGEN, USA) containing buffer, dNTPs, and a thermally-activatable *Taq* DNA polymerase. This solution is placed in a thermal cycler, heated to 95°C for 15 minutes to activate the polymerase and cooled to 70°C for 5 minutes to allow the primers to anneal to DNA inserts and extend into the forward adapter site.

[00104] Amplification

Amplification primers that are complementary to the 5' ends of the forward and reverse adapter sites but also contain portions complementary to flow cell oligonucleotide sequences are added to the 16 individual primer extension products (above). Selection for fragments that contain both the forward and reverse adapter, and the respective universal priming sites, is accomplished with PCR using a 3-step temperature routine (94°C for 30 seconds, 60°C for 30 seconds, 72° C for 1 minute) for 25 cycles. PCR products are purified using AMPure XP beads and analyzed with a 2100 Bioanalyzer (Agilent Technologies).

[00105] Sequencing and data analysis

Equal masses of each of the amplified libraries (above) are pooled and diluted to working concentrations according to manufacturer's recommendations. Single end sequencing reads of 100 nt length are obtained for libraries using a MiSeq System (Illumina). Raw sequencing data is processed using Illumina base calling software. Samples from the various time points are binned based on their unique 8 base code and mapped to a reference database. Based on the mapping characteristics, individual samples or a new pool of samples can be rerun on the sequencer to obtain greater read depth. Samples with poor gene coverage will be eliminated from the pool.

CLAIMS**WHAT IS CLAIMED IS:**

1. A method for enriching for target nucleic acid sequences of interest in a sample comprising nucleic acids, the method comprising:
 - a. fragmenting the nucleic acids, thereby generating nucleic acid fragments;
 - b. appending a first adaptor to a 5' end of each nucleic acid fragment;
 - c. annealing one or more oligonucleotides to the nucleic acid fragments, whereby each of the one or more oligonucleotides comprise a 3' portion that is complementary to a target nucleic acid sequence of interest present in one or more of the nucleic acid fragments, and a 5' portion comprising a second adaptor sequence;
 - d. extending the one or more oligonucleotides with a polymerase thereby generating one or more oligonucleotide extension products with the first adaptor at a first end and the second adaptor sequence at a second end; and
 - e. amplifying the one or more oligonucleotide extension product using a first primer that is complementary to the first adaptor and a second primer that is complementary to the second adaptor sequence to enrich for nucleic acid fragments containing the first adaptor and the second adaptor sequence at each end.
2. The method of claim 1, further comprising an additional step of sequencing the one or more oligonucleotide extension product following amplification.
3. The method of claim 1, wherein the target nucleic acid sequences of interest comprise genomic DNA, RNA, or cDNA.
4. The method of claim 1, wherein the target nucleic acid sequences of interest comprise genomic DNA.
5. The method of claim 1, wherein the target nucleic acid sequences of interest comprise cDNA.
6. The method of claim 1, further comprising denaturing the nucleic acid fragments prior to step c, thereby generating single-stranded nucleic acid fragments with the first adaptor sequence at the 5' end.

7. The method of claim 1, wherein the first adaptor is common to each nucleic acid fragment.
8. The method of claim 1, wherein the second adaptor sequence is common to the one or more oligonucleotides.
9. The method of claim 1, wherein the first adaptor and the second adaptor sequence are distinct from each other.
10. The method of claim 1, wherein the first adaptor and/or the second adaptor sequence further comprise barcode sequence.
11. The method of claim 1, wherein step b is performed by ligation.
12. The method of claim 1, further comprising an additional step of performing gap repair following ligation of the first adapter to create nucleic acid fragments with complementary termini.
13. A composition comprising the target nucleic acid sequences of interest enriched by the method of claim 1.
14. The method of claim 1, wherein the polymerase is a DNA polymerase.
15. A method for enriching for target nucleic acid sequences of interest in a sample comprising nucleic acids, the method comprising:
 - a. fragmenting the nucleic acids, thereby generating nucleic acid fragments;
 - b. appending a first adapter to the nucleic acid fragments wherein the first adaptor comprises a partial duplex with a short strand and a long strand wherein a 3' end of the short strand of the partial duplex adaptor comprises a blocking group, and a 5' end of the long strand of the partial duplex adaptor comprises a restriction and/or cleavage site for a nucleic acid modifying enzyme;
 - c. denaturing the nucleic acid fragments, thereby creating single-stranded nucleic acid fragments;
 - d. annealing one or more oligonucleotides to the single stranded nucleic acid fragments, whereby each of the one or more oligonucleotides comprise sequence that is complementary to a target nucleic acid sequence of interest present in one or more of the single-stranded nucleic acid fragments, extending the one or more oligonucleotides with a polymerase

- to produce one or more double stranded nucleic acid complexes comprising the target nucleic acid sequences and their complements, a first end with a double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme, and a second end with a 3' overhang comprising the short strand of the first adaptor;
- e. cleaving the double stranded restriction and/or cleavage site with the nucleic acid modifying enzyme, thereby generating a cleavage site;
 - f. ligating a second adaptor to the cleavage site, wherein the second adaptor comprises a duplex with two strands;
 - g. denaturing the one or more double-stranded nucleic acid complexes, thereby generating one or more single stranded nucleic acid fragments comprising the target nucleic acid sequences, a strand from the second adapter at the first end, and the short strand of the first adaptor at the second end; and
 - h. amplifying the one or more single stranded nucleic acid fragments comprising the one or more target nucleic acid sequences with a first primer comprising sequence complementary to the strand from the second adapter and a second primer comprising sequence complementary to the short strand of the first adaptor, thereby enriching for the one or more target nucleic acid sequences.
16. The method of claim 15, further comprising an additional step of sequencing the one or more single stranded nucleic acid fragments from step h following amplification.
17. The method of claim 15, wherein the target nucleic acid sequences of interest comprise genomic DNA, RNA, or cDNA.
18. The method of claim 15, wherein the target nucleic acid sequences of interest comprise genomic DNA
19. The method of claim 15, wherein the target nucleic acid sequences of interest comprise cDNA.
20. The method of claim 15, wherein the first adaptor and the second adaptor are common to each of the nucleic acid fragments.

21. The method of claim 15, wherein the first adaptor and the second adaptor are distinct from each other.
22. The method of claim 15, wherein the first adaptor and/or the second adaptor further comprise barcode sequence.
23. The method of claim 15, wherein the double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme from step e comprises the 5' end of the long strand of the partial duplex of the first adaptor and sequence complementary to the 5' end of the long strand of the partial duplex of the first adaptor generated from extension of the one or more oligonucleotides;
24. The method of claim 15, wherein the nucleic acid modifying enzyme comprises a restriction enzyme.
25. The method of claim 15, wherein step b is performed by ligation.
26. A composition comprising the target nucleic sequences of interest enriched by the method of claim 15.
27. The method of claim 15, wherein the polymerase is a DNA polymerase.
28. A method for generating a library of nucleic acid sequences, the method comprising:
 - a. fragmenting a sample comprising nucleic acids, thereby generating nucleic acid fragments;
 - b. appending a first adapter to each of the nucleic acid fragments;
 - c. denaturing the nucleic acid fragments, thereby generating a library of single-stranded nucleic acid fragments;
 - d. annealing one or more oligonucleotides to the single-stranded nucleic acid fragments wherein each of the one or more oligonucleotides comprises a 3' portion complementary to sequence in one or more of the single-stranded nucleic acid fragments and a 5' portion comprising a second adaptor sequence;
 - e. extending the one or more oligonucleotides with a polymerase thereby generating one or more oligonucleotide extension products comprising the first adaptor at a first end and the second adapter sequence at a second end;and

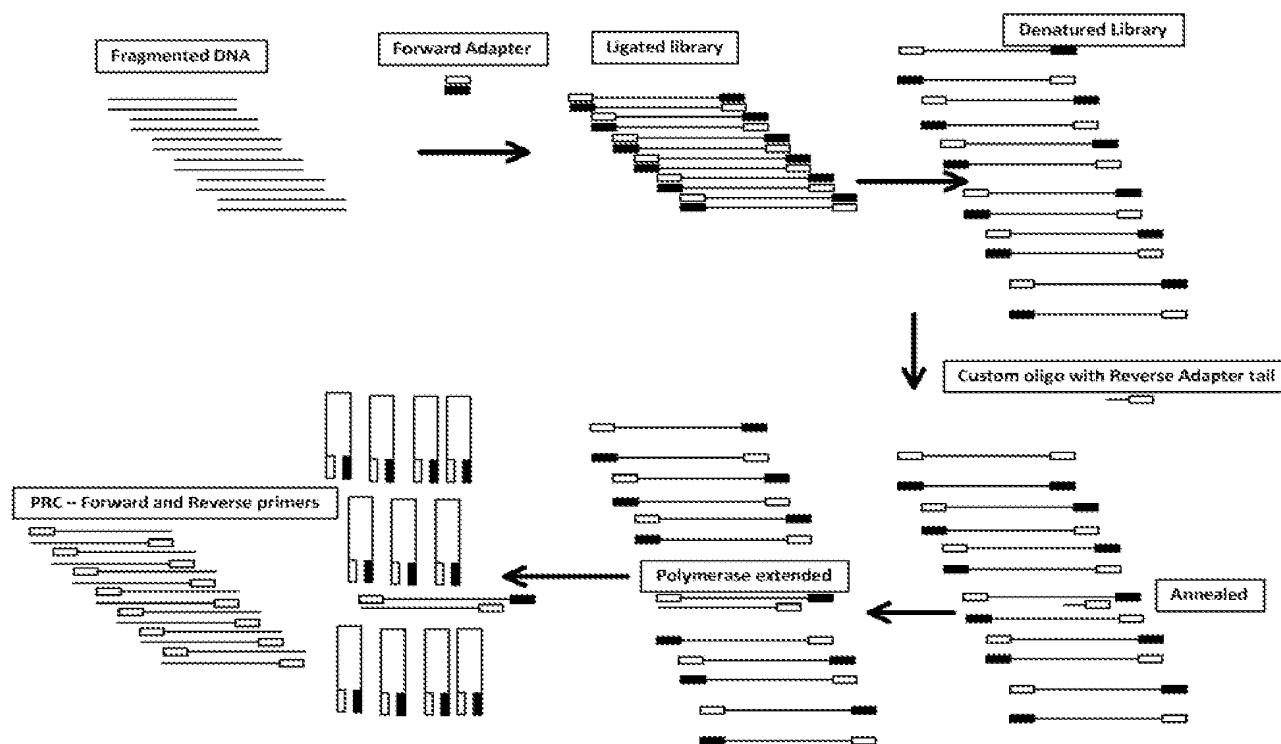
- f. amplifying the one or more oligonucleotide extension products with a set of primers specific to the first adaptor and the second adaptor sequence to generate a library of nucleic acid fragments comprising the first adaptor and second adaptor sequence at each end.
29. The method of claim 28, further comprising an additional step of performing a gap repair reaction following ligation of the forward adapter to create nucleic acid fragments with complementary termini.
30. The method of claim 28, further comprising an additional step of sequencing the amplified one or more oligonucleotide extension product from step f.
31. The method of claim 28, wherein the nucleic acid sequence comprises genomic DNA.
32. The method of claim 28, wherein the nucleic acid sequence comprises cDNA.
33. The method of claim 28, without step c, wherein the nucleic acid fragments are double-stranded.
34. The method of claim 28, wherein the 3' portion of the one or more oligonucleotides of step d comprises random sequence.
35. The method of claim 28, wherein step b is performed by ligation.
36. The method of claim 28, wherein the first adaptor and the second adaptor sequence are common to each nucleic acid fragment.
37. The method of claim 28, wherein the first adaptor and the second adaptor sequence are distinct from each other.
38. The method of claim 28, wherein the first adaptor and/or the second adaptor sequence further comprise barcode sequence.
39. A composition comprising the library of nucleic sequences generated by the method of claim 28.
40. The method of claim 28, wherein the polymerase is a DNA polymerase.
41. A method for enriching for target nucleic acid sequences of interest from a library comprising nucleic acid inserts with a first adaptor on a first end and a second adaptor on a second end, the method comprising:

- a. denaturing the nucleic acid inserts, thereby generating a library of single stranded nucleic acid inserts;
 - b. annealing one or more oligonucleotides to the single stranded nucleic acid inserts, wherein each of the one or more oligonucleotides comprises a 3' portion that is complementary to a target nucleic acid sequence of interest present in one or more of the nucleic acid inserts, and a 5' portion comprising a third adaptor sequence;
 - c. extending the one or more oligonucleotides with a polymerase thereby generating one or more oligonucleotide extension products with the first adaptor at the first end and the third adaptor sequence at the second end; and
 - d. amplifying the one or more oligonucleotide extension products using a first primer that is complementary to the first adaptor and a second primer that is complementary to the third adaptor sequence to enrich for nucleic acid fragments containing the first adaptor and the third adaptor sequence at each end.
42. The method of claim 41, further comprising an additional step of sequencing the amplified one or more oligonucleotide extension products from step d.
43. The method of claim 41, wherein the target nucleic acid sequences of interest comprises genomic DNA.
44. The method of claim 41, wherein the target nucleic acid sequences of interest comprises cDNA.
45. The method of claim 41, without step a, wherein the nucleic acid fragments are double-stranded.
46. The method of claim 41, wherein the first adaptor and the second adaptor are common to each nucleic acid fragment.
47. The method of claim 41, wherein the third adaptor sequence is common to the one or more oligonucleotides.
48. The method of claim 41, wherein the first adaptor and the second adaptor are distinct from each other.

49. The method of claim 41, wherein the first adaptor and the second adaptor are the same.
50. The method of claim 41, wherein the first adaptor, the second adaptor and/or the third adaptor sequence further comprise barcode sequence.
51. A composition comprising the target nucleic acid sequences of interest enriched by the method of claim 41.

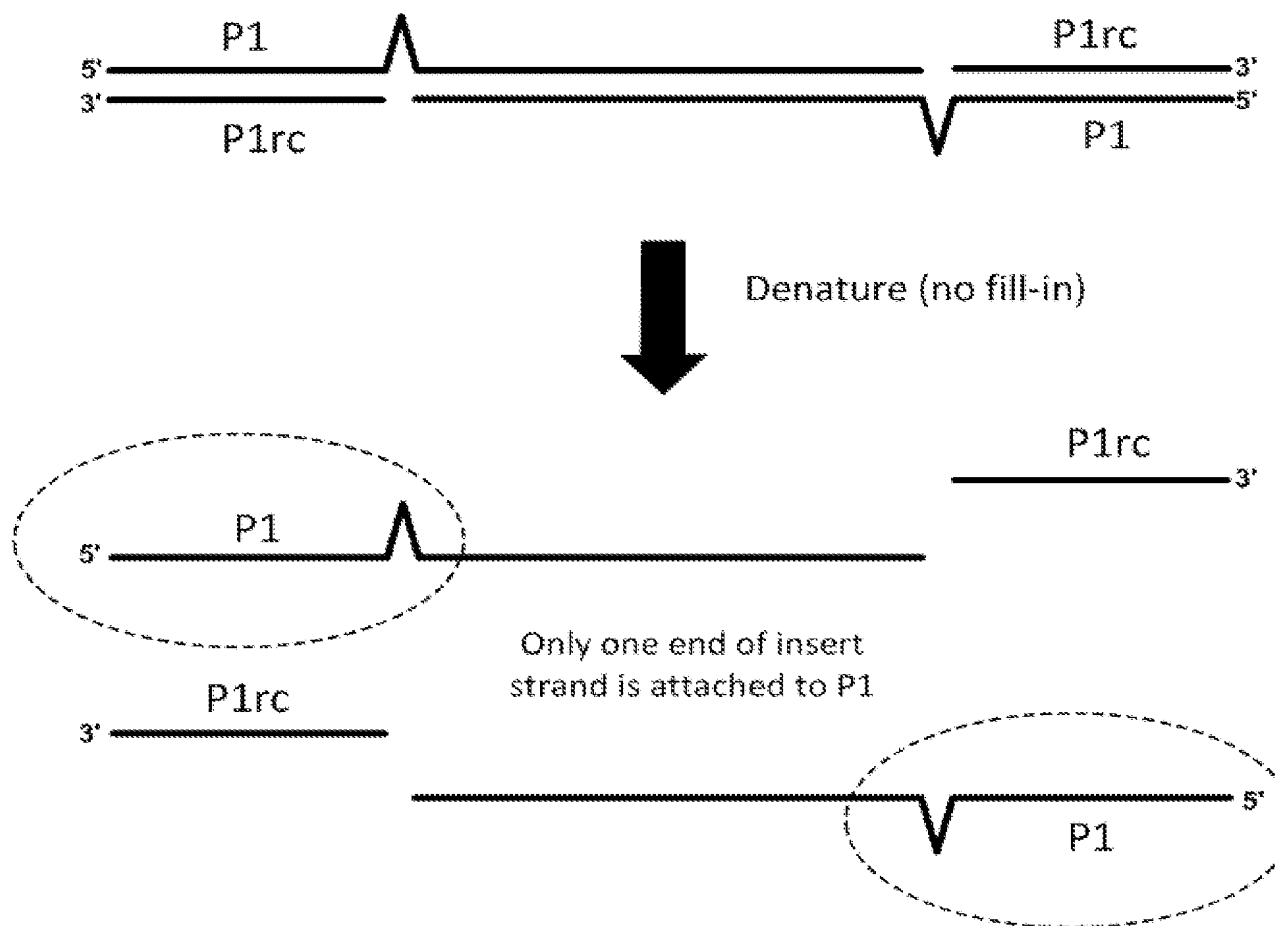
FIG. 1

Subtractive PRC Enrichment



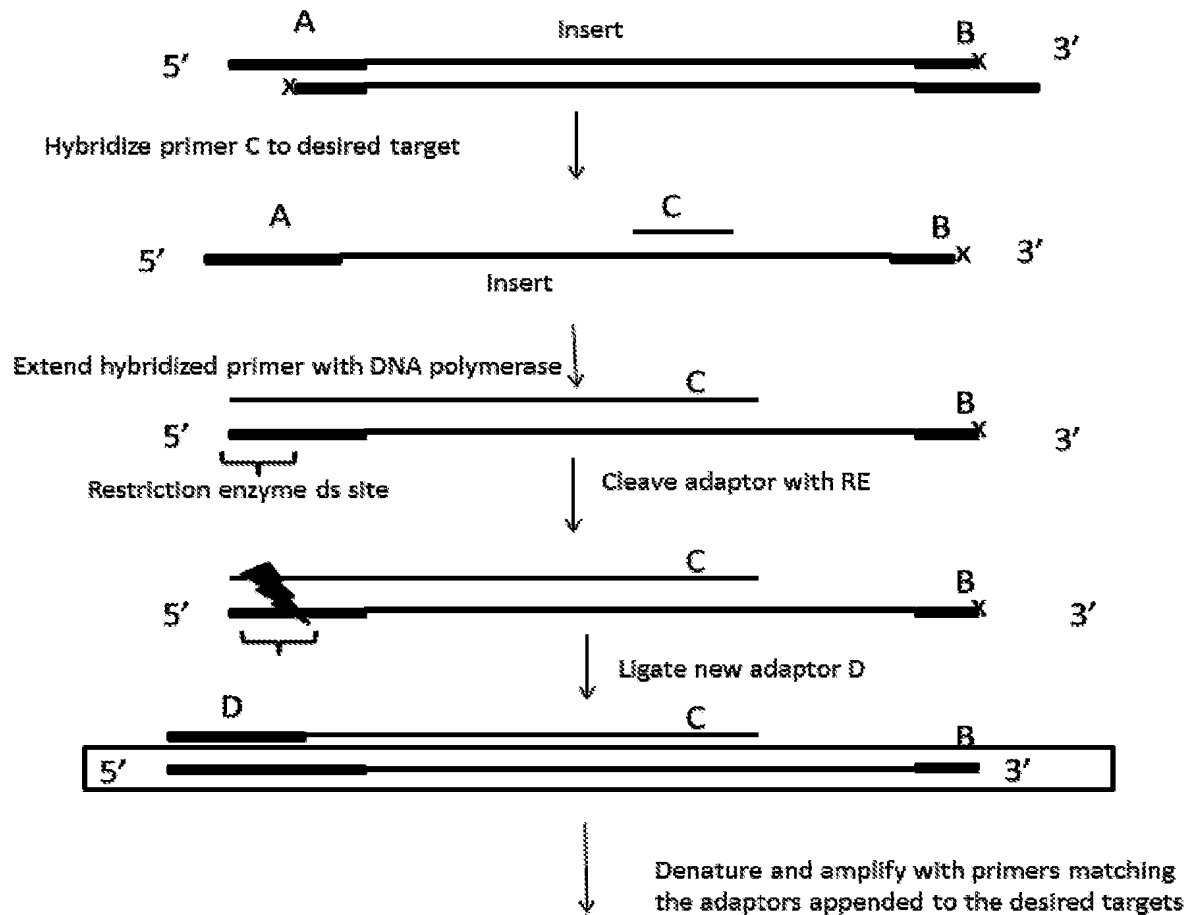
SUBSTITUTE SHEET (RULE 26)

FIG. 2



SUBSTITUTE SHEET (RULE 26)

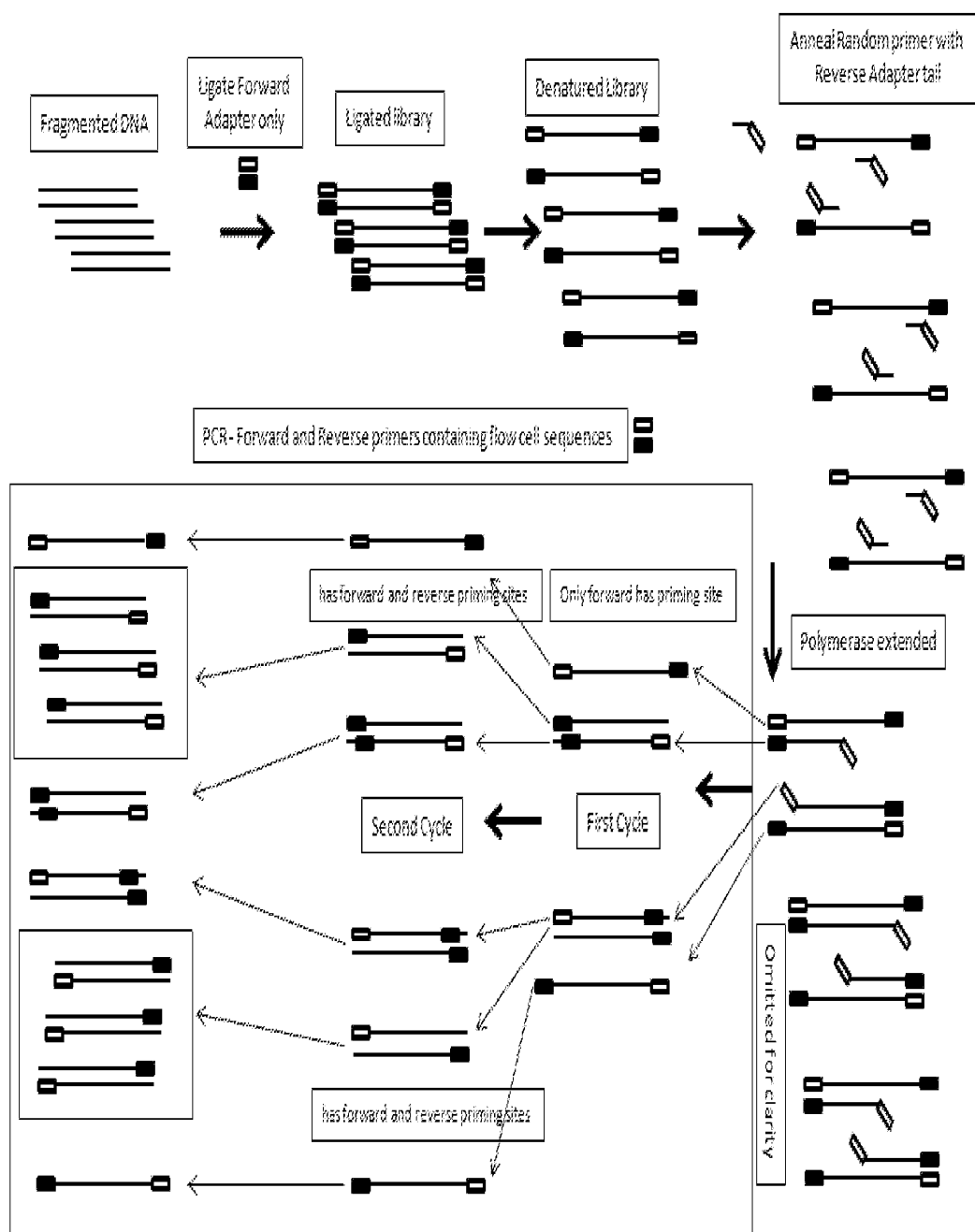
FIG. 3

Primer Directed Targeted Enrichment Of Sequences Of Interest In A Complex Library

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FIG. 4

High Efficiency Library Generation



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/023278

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C40B 50/06 (2013.01)

USPC - 506/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C12P 19/34; C12Q 1/68; C40B 40/06, 40/08, 50/06 (2013.01)

USPC - 435/91.2, 91.5, 91.51, 91.52, 91.53; 506/16, 17, 26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12N 15/10; C12P 19/34; C12Q 1/6806, 1/6844, 1/6853, 1/6855 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit.com, Google Patents, Google Scholar, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BORODINA et al. 'A Strand-Specific Library Preparation Protocol for RNA Sequencing Methods in Enzymology,' Methods in Enzymology, Volume 500 Pages 79-98 (2011). entire document	1-9, 11-14, 28-37, 39-48, 51
Y		10, 15-27, 38, 50
Y	US 2011/0104785 A1 (VAIDYANATHAN et al) 05 May 2011 (05.05.2011) entire document	10, 15-27, 38, 50
Y	US 2007/0231823 A1 (MCKERNAN et al) 04 October 2007 (04.10.2007) entire document	15-27
A	LEVIN et al. 'Comprehensive comparative analysis of strand-specific RNA sequencing methods nature methods,' Nature Methods, VOL.7 NO.9 SEPTEMBER 2010 Pages. 709-718. entire document	1-51

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 March 2013

Date of mailing of the international search report

16 APR 2013

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Authorized officer:

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

IDT Exhibit 1017.2365

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 25115-755601	FOR FURTHER ACTION		see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US2013/023278	International filing date (<i>day/month/year</i>) 25 January 2013	(Earliest) Priority Date (<i>day/month/year</i>) 26 January 2012	
Applicant NUGEN TECHNOLOGIES, INC.			

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. 1

☒ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

Form PCT/ISA/210 (first sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/023278

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C40B 50/06 (2013.01)

USPC - 506/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C12P 19/34; C12Q 1/68; C40B 40/06, 40/08, 50/06 (2013.01)

USPC - 435/91.2, 91.5, 91.51, 91.52, 91.53; 506/16, 17, 26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC - C12N 15/10; C12P 19/34; C12Q 1/6806, 1/6844, 1/6853, 1/6855 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit.com, Google Patents, Google Scholar, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y		10, 15-27, 38, 50
Y	US 2011/0104785 A1 (VAIDYANATHAN et al) 05 May 2011 (05.05.2011) entire document	10, 15-27, 38, 50
Y	US 2007/0231823 A1 (MCKERNAN et al) 04 October 2007 (04.10.2007) entire document	15-27
A	LEVIN et al. 'Comprehensive comparative analysis of strand-specific RNA sequencing methods nature methods,' Nature Methods, VOL.7 NO.9 SEPTEMBER 2010 Pages. 709-718. entire document	1-51

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 March 2013

Date of mailing of the international search report

16 APR 2013

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: MAYA SKUBATCH
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing
(day/month/year)

16 APR 2013

Applicant's or agent's file reference
25115-755601

FOR FURTHER ACTION

See paragraph 2 below

International application No.
PCT/US2013/023278

International filing date (day/month/year)
25 January 2013

Priority date (day/month/year)
26 January 2012

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C40B 50/06 (2013.01)
USPC - 506/26

Applicant NUGEN TECHNOLOGIES, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis. 1(a)(i) with regard to novelty, inventive step or industrial applicability: citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Date of completion of this opinion
26 March 2013

Authorized officer:
Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITYInternational application No.
PCT/US2013/023278

Box No. 1 Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:
- ☒ the international application in the language in which it was filed.
- ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))
3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:
- a. (means)
- ☐ on paper
- ☐ in electronic form
- b. (time)
- ☐ in the international application as filed
- ☐ together with the international application in electronic form
- ☐ subsequently to this Authority for the purposes of search
4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2013/023278

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	10, 12, 15-27, 29, 38, 49, 50	YES
	Claims	1-9, 11, 13, 14, 28, 30-37, 39-48, 51	NO
Inventive step (IS)	Claims	49	YES
	Claims	1-48, 50, 51	NO
Industrial applicability (IA)	Claims	1-51	YES
	Claims	None	NO

2. Citations and explanations:

Claims 1-9, 11, 13, 14, 28, 30-37, 39-48, and 51 lack novelty under PCT Article 33(2) as being anticipated by Borodina et al. (hereafter Borodina).

Regarding claim 1, Borodina discloses a method for enriching for target nucleic acid sequences of interest in a sample comprising nucleic acids (pg. 83, Para. 2, detailed protocol for the preparation of an ssRNA-Seq library from mRNA. The next steps enable the ligation of adaptor sequences on both ends of the ds cDNAs) the method comprising:

a. fragmenting the nucleic acids, thereby generating nucleic acid fragments (Pg. 83, Para. 3, Day 1: RNA fragmentation.);
b. appending a first adaptor to a 5' end of each nucleic acid fragment (Pg. 83 Para. 3, Day 3: adapter ligation; Figure 5.1, showing ligation of Y-adaptors ligated to 5'ends; Pg. 93, Para. 2, PE genomic adaptors; Pg. 94, 2.4.4. Adapter ligation, PE genomic adapter...we first mix the cDNA with the adapters, then mix thoroughly with the buffer, and then add ligase); c. annealing one or more oligonucleotides to the nucleic acid fragments, whereby each of the one or more oligonucleotides comprise a 3' portion that is complementary to a target nucleic acid sequence of interest present in one or more of the nucleic acid fragments (Pg. 96, 2.4.8. Amplification of the library, amplification reaction mix...0.4 mM oligos #PE PCR primer 1.1 and #PE PCR primer 2.1), and a 5' portion comprising a second adaptor sequence (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5'ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3', # adapter primer 1: 5'-ACACTCTTCCCTACACGACGCTCTTCCGATCT-3');
d. extending the one or more oligonucleotides with a polymerase thereby generating one or more oligonucleotide extension products with the first adaptor at a first end and the second adaptor sequence at a second end (Pg. 96, 2.4.8. Amplification of the library, Prepare the amplification reaction mix, Phusion HF Master Mix, 0.4 mM oligos #PE PCR primer 1.1 and #PE PCR primer 2.1. Take 10-30 ml of the library for the reaction...Place the reaction in a PCR machine and run the following program (thus extending the oligonucleotide primers));
and e. amplifying the one or more oligonucleotide extension product using a first primer that is complementary to the first adaptor and a second primer that is complementary to the second adaptor sequence to enrich for nucleic acid fragments containing the first adaptor and the second adaptor sequence at each end (Pg. 96, 2.4.8. Amplification of the library, Prepare the amplification reaction mix, Phusion HF Master Mix, 0.4 mM oligos #PE PCR primer 1.1 and #PE PCR primer 2.1. Take 10-30 ml of the library for the reaction...Place the reaction in a PCR machine and run the following program (thus extending the oligonucleotide primers and amplifying the extension products)).

Regarding claim 28, Borodina discloses a method for generating a library of nucleic acid sequences (Pg. 83, Para. 2, detailed protocol for the preparation of an ssRNA-Seq library from mRNA), the method comprising:

a. fragmenting a sample comprising nucleic acids, thereby generating nucleic acid fragments (Pg. 83, Para. 3, Day 1: RNA fragmentation);
b. appending a first adapter to each of the nucleic acid fragments (Pg. 83 Para. 3, Day 3: adapter ligation; Figure 5.1, showing ligation of Y-adaptors ligated to 5'ends; Pg. 93, Para. 2, PE genomic adaptors; Pg. 94, 2.4.4. Adapter ligation, PE genomic adapter...we first mix the cDNA with the adapters, then mix thoroughly with the buffer, and then add ligase); c. denaturing the nucleic acid fragments, thereby generating a library of single-stranded nucleic acid fragments (Pg. 96, 2.4.8. Amplification of the library, 2. Place the reaction in a PCR machine and run the following program: 95 C for 30 s (i.e. denaturation step)); d. annealing one or more oligonucleotides to the single-stranded nucleic acid fragments (Pg. 96, 2.4.8. Amplification of the library, 2. Place the reaction in a PCR machine and run the following program: 95 C for 30 s, followed by cycles of: 95C for 15 s, 65C for 30 s (annealing step), 72 C for 30 s.); wherein each of the one or more oligonucleotides comprises a 3' portion complementary to sequence in one or more of the single-stranded nucleic acid fragments and a 5' portion comprising a second adaptor sequence (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5'ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3', # adapter primer 1: 5'-ACACTCTTCCCTACACGACGCTCTTCCGATCT-3');
e. e. extending the one or more oligonucleotides with a polymerase thereby generating one or more oligonucleotide extension products comprising the first adaptor at a first end and the second adaptor sequence at a second end (Pg. 96, 2.4.8. Amplification of the library, 1. Prepare the amplification reaction mix in a 0.2 ml thin-wall tube: 1X Phusion HF Master Mix (comprises polymerase), 0.4 mM oligos #PE PCR primer 1.1 and #PE PCR primer 2.1. Take 10-30 ml of the library for the reaction. 2. Place the reaction in a PCR machine and run the following program: 95 C for 30 s, followed by cycles of: 95C for 15 s, 65C for 30 s, 72 C for 30 s.); and
f. amplifying the one or more oligonucleotide extension products with a set of primers specific to the first adaptor and the second adaptor sequence to generate a library of nucleic acid fragments comprising the first adaptor and second adaptor sequence at each end (Pg. 96, 2.4.8. Amplification of the library, Prepare the amplification reaction mix, Phusion HF Master Mix, 0.4 mM oligos #PE PCR primer 1.1 and #PE PCR primer 2.1. Take 10-30 ml of the library for the reaction...Place the reaction in a PCR machine and run the following program (thus extending the oligonucleotide primers and amplifying the extension products)).

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2013/023278

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding claim 41, Borodina discloses a method for enriching for target nucleic acid sequences of interest from a library (Pg. 83, Para. 2, detailed protocol for the preparation of an ssRNA-Seq library from mRNA) comprising nucleic acid inserts with a first adaptor on a first end and a second adaptor on a second end (Pg. 83 Para. 3, Day 3: adapter ligation; Figure 5.1, showing ligation of Y-adaptors ligated to first and second ends; Pg. 93, Para. 2, PE genomic adapters; Pg. 94, 2.4.4. Adapter ligation, PE genomic adapter...we first mix the cDNA with the adapters, then mix thoroughly with the buffer, and then add ligase), the method comprising:

a. denaturing the nucleic acid inserts, thereby generating a library of single stranded nucleic acid inserts (Pg. 96, 2.4.8. Amplification of the library, 2. Place the reaction in a PCR machine and run the following program: 95 C for 30 s (i.e. denaturation step); Pg. 88, Place the sample at 65 degrees C for 2 min to denature the secondary structure); b. annealing one or more oligonucleotides to the single stranded nucleic acid inserts (Pg. 96, 2.4.8. Amplification of the library, 2. Place the reaction in a PCR machine and run the following program: 95 C for 30 s, followed by cycles of: 95C for 15 s, 65C for 30 s (annealing step), 72 C for 30 s.), wherein each of the one or more oligonucleotides comprises a 3' portion that is complementary to a target nucleic acid sequence of interest present in one or more of the nucleic acid inserts, and a 5' portion comprising a third adaptor sequence (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5'ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3', # adapter primer 1: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'); c. extending the one or more oligonucleotides with a polymerase thereby generating one or more oligonucleotide extension products with the first adaptor at the first end and the third adaptor sequence at the second end (Pg. 96, 2.4.8. Amplification of the library, Prepare the amplification reaction mix, Phusion HF Master Mix, 0.4 mM oligos #PE PCR primer 1.1 and #PE PCR primer 2.1. Take 10-30 ml of the library for the reaction...Place the reaction in a PCR machine and run the following program (thus extending the oligonucleotide primers)); and d. amplifying the one or more oligonucleotide extension products using a first primer that is complementary to the first adaptor and a second primer that is complementary to the third adaptor sequence to enrich for nucleic acid fragments containing the first adaptor and the third adaptor sequence at each end (Pg. 96, 2.4.8. Amplification of the library, Prepare the amplification reaction mix, Phusion HF Master Mix, 0.4 mM oligos #PE PCR primer 1.1 and #PE PCR primer 2.1. Take 10-30 ml of the library for the reaction...Place the reaction in a PCR machine and run the following program (thus extending the oligonucleotide primers and amplifying the extension products)).

Regarding claim 2, Borodina discloses the method of claim 1. Borodina further discloses an additional step of sequencing the one or more oligonucleotide extension product following amplification (Pg. 83, para. 2, an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing; Figure 5.2, showing sequencing after library amplification).

Regarding claim 3, Borodina discloses the method of claim 1. Borodina further discloses wherein the target nucleic acid sequences of interest comprise genomic DNA, RNA, or cDNA (Pg. 83, Paras. 2, 3, preparation of an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing. The initial step of this part is to generate a cDNA molecule).

Regarding claims 4, 31, and 43, Borodina discloses the method of claims 1, 28, and 41. Borodina further discloses wherein the target nucleic acid sequences of interest comprise genomic DNA (Pg. 92, Para. 3, based on the original Illumina protocol of library construction from genomic DNA).

Regarding claims 5, 32, and 44, Borodina discloses the method of claims 1, 28, and 41. Borodina further discloses wherein the target nucleic acid sequences of interest comprise cDNA (Pg. 83, Paras. 2, 3, preparation of an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing. The initial step of this part is to generate a cDNA molecule).

Regarding claim 6, Borodina discloses the method of claim 1. Borodina further discloses denaturing the nucleic acid fragments prior to step c, thereby generating single-stranded nucleic acid fragments with the first adaptor sequence at the 5' end (Pg. 96, 2.4.8. Amplification of the library, 2. Place the reaction in a PCR machine and run the following program: 95 C for 30 s (i.e. denaturation step); PCR primer 1.1 and PCR primer 2.1 (the primers prime synthesis at sites provided on the adaptors on the 5' ends)).

Regarding claim 7, Borodina discloses the method of claim 1. Borodina further discloses wherein the first adaptor is common to each nucleic acid fragment (Pg. 94, 2.4.4. Adapter ligation, Prepare the reaction mix in a 1.5-ml LoBind tube: 10 ml of ds cDNA, PE genomic adapter (the same adapter is ligated to all the fragments)).

Regarding claim 8, Borodina discloses the method of claim 1. Borodina further discloses wherein the second adaptor sequence is common to the one or more oligonucleotides (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5'ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3', # adapter primer 1: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' (the ligated adaptors comprise the sequence CGCTCTTCCGATCT-3', common to the amplifying oligonucleotides).

Regarding claims 9, and 37, Borodina discloses the method of claims 1 and 28. Borodina further discloses wherein the first adaptor and the second adaptor sequence are distinct from each other (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5'ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3', # adapter primer 1: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' (after amplification with adapter primers, the 5' ends have been adapted to comprise a primer binding site suitable for sequencing with Illumina primers); Pg. 83, para. 2, an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing).

Regarding claims 11 and 35, Borodina discloses the method of claims 1 and 28. Borodina further discloses wherein step b is performed by ligation (Pg. 83 Para. 3, Day 3: adapter ligation; Figure 5.1, showing ligation of Y-adaptors ligated to 5'ends; Pg. 93, Para. 2, PE genomic adapters; Pg. 94, 2.4.4. Adapter ligation, PE genomic adapter...we first mix the cDNA with the adapters, then mix thoroughly with the buffer, and then add ligase).

Regarding claims 13, 39, and 51, Borodina discloses a composition comprising the target nucleic acid sequences of interest enriched by the method of claims 1, 28, and 41 (Pg. 96, Para. 2, Load the entire PCR reaction on an agarose gel and excise cDNAs ranging in size between 230 and 270 bp...Proceed to the flowcell preparation, cluster generation, and sequencing reaction on the Illumina Cluster Station).

Form PCT/ISA/237 (Supplemental Box) (July 2011)

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2013/023278

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding claims 14 and 40, Borodina discloses the method of claims 1 and 28. Borodina further discloses wherein the polymerase is a DNA polymerase (Pg. 96, Prepare the amplification reaction mix in a 0.2 ml thin-wall tube: 1X Phusion HF Master Mix (Phusion HF is a high fidelity thermostable DNA polymerase).

Regarding claim 30, Borodina discloses the method of claim 28. Borodina further discloses an additional step of sequencing the amplified one or more oligonucleotide extension product from step f (Pg. 83, para. 2, an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing; Figure 5.2, showing sequencing after library amplification).

Regarding claims 33 and 45, Borodina discloses the method of claims 28 and 41, without step c (claim 28) and step a (claim 41), wherein the nucleic acid fragments are double-stranded (Pg. 80, para. 1, The ds cDNA fragments are further processed following a standard sequencing library preparation scheme tailored for the Illumina sequencing platform; end polishing, A-tailing, adapter ligation, and size selection; Pg. 92, Para. 3, The procedure of sequencing library preparation for Illumina sequencing platform that we describe below is based on the original Illumina protocol of library construction from genomic DNA (i.e. ligation of adapters to double stranded fragments without an initial denaturation step)).

Regarding claim 34, Borodina discloses the method of claim 28. Borodina further discloses wherein the 3' portion of the one or more oligonucleotides of step d comprises random sequence (Figure 5.2, Random hexamer primed first strand synthesis).

Regarding claims 36 and 46, Borodina discloses the method of claims 28 and 41. Borodina further discloses wherein the first adaptor and the second adaptor sequence are common to each nucleic acid fragment (Pg. 94, 2.4.4. Adapter ligation, Prepare the reaction mix in a 1.5-ml LoBind tube: 10 ml of ds cDNA, PE genomic adapter (the same adapter is ligated to all the fragments), then adapter primers are used to create fragments comprising first (3' segment) and second (priming site for sequencing primer in 5' segment) adapter segments).

Regarding claim 42, Borodina discloses the method of claim 41. Borodina further discloses an additional step of sequencing the amplified one or more oligonucleotide extension products from step d (Pg. 83, para. 2, an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing; Figure 5.2, showing sequencing after library amplification).

Regarding claim 47, Borodina discloses the method of claim 41. Borodina further discloses wherein the third adaptor sequence is common to the one or more oligonucleotides (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5' ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3', # adapter primer 1: 5'-ACACTCTTCCCTACACGACGCTCTTCCGATCT-3' (the ligated adaptors comprise the sequence CGTCTTCCGATCT-3', common to the amplifying oligonucleotides (3' end of the adapter primer oligonucleotides comprising the third adapter)).

Regarding claim 48, Borodina discloses the method of claim 41. Borodina further discloses wherein the first adaptor and the second adaptor are distinct from each other (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5' ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3', # adapter primer 1: 5'-ACACTCTTCCCTACACGACGCTCTTCCGATCT-3 (after amplification with adapter primers, the 5' ends (i.e. second adapter) have been adapted to comprise a primer binding site suitable for sequencing with Illumina primers); Pg. 83, para. 2, an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing).

Claims 12 and 29 lack an inventive step under PCT Article 33(3) as being obvious over Borodina et al. (hereafter Borodina).

Regarding claims 12 and 29, Borodina discloses the method of claims 1 and 28. Borodina further discloses a step of performing gap repair to create nucleic acid fragments with complementary termini (Pg. 94, 2.4.2. End-repair, 2.4.3. A-Tailing, 2.4.4. Adapter ligation (gaps at the fragment ends are filled and A-tailed to provide a complementary end for ligation of a protruding T on the adapter). Borodina fails to explicitly disclose gap repair following ligation of the first adapter.

It would have been obvious to one of ordinary skill in the art at the time the invention was made to perform additional gap repair steps, since selection of any order of process steps which did not result in a new or unexpected result would involve only routine skill in the art. The motivation for doing so would be to provide suitable termini on the fragments for further processing as taught by Borodina (Pg. 81, Para. 4, the common strategy of an RNA-Seq library preparation was to convert single-stranded RNA molecules into double-stranded (ds) DNA fragments of a certain size flanked by platform-specific adapter sequences... Several strand-specific RNA-Seq (ssRNA-Seq) protocols based either on the ligation of adapters to the RNA molecules or on modifications of the ds cDNA synthesis procedure were suggested recently).

Claims 10, 38, and 50 lack an inventive step under PCT Article 33(3) as being obvious over Borodina et al. (hereafter Borodina) in view of Vaidyanathan et al. (hereafter Vaidyanathan).

Regarding claim 10, Borodina discloses the method of claim 1. Borodina fails to explicitly disclose wherein the first adaptor and/or the second adaptor sequence further comprise barcode sequences. Vaidyanathan teaches RNA/cDNA libraries (Para. [0024], The present invention also provides kits for preparing end tagged and dual end-tagged RNA or ds cDNA libraries, suitable for applications such as next-generation sequencing, qPCR, microarray analysis, or cloning) appended with adaptors comprising barcode sequences (Para. [0020], 3' tagging of RNA with barcoded oligonucleotides for RNA-Seq).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Borodina to comprise barcoded adaptors as taught by Vaidyanathan. The motivation would be to use an established method for identifying sequencing templates as taught by Vaidyanathan (Para. [0019], commercial synthesis of such 5'- and 3'-modified oligonucleotides can be inefficient and expensive compared to standard (unmodified) oligonucleotides, especially when many oligonucleotides are required for applications such as preparing barcoded libraries for RNA-Seq).

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In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding claim 38, Borodina discloses the method of claim 28. Borodina fails to explicitly disclose wherein the first adaptor and/or the second adaptor sequence further comprise barcode sequences. Vaidyanathan teaches RNA/cDNA libraries (Para. [0024]. The present invention also provides kits for preparing end tagged and dual end-tagged RNA or ds cDNA libraries, suitable for applications such as next-generation sequencing, qPCR, microarray analysis, or cloning) appended with adaptors comprising barcode sequences (Para. [0020], 3' tagging of RNA with barcoded oligonucleotides for RNA-Seq).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Borodina to comprise barcoded adaptors as taught by Vaidyanathan. The motivation would be to use an established method for identifying sequencing templates as taught by Vaidyanathan (Para. [0019], commercial synthesis of such 5'- and 3'-modified oligonucleotides can be inefficient and expensive compared to standard (unmodified) oligonucleotides, especially when many oligonucleotides are required for applications such as preparing barcoded libraries for RNA-Seq (Vigneault, 2008); [0058], the methods provide rapid, efficient, and high throughput preparation of oligonucleotides suitable for applications such as barcoding RNA samples for RNA-Seq.).

Regarding claim 50, Borodina discloses the method of claim 41. Borodina fails to explicitly disclose wherein the first adaptor, the second adaptor and/or the third adaptor sequence further comprise barcode sequence. Vaidyanathan teaches RNA/cDNA libraries (Para. [0024]. The present invention also provides kits for preparing end tagged and dual end-tagged RNA or ds cDNA libraries, suitable for applications such as next-generation sequencing, qPCR, microarray analysis, or cloning) appended with adaptors comprising barcode sequences (Para. [0020], 3' tagging of RNA with barcoded oligonucleotides for RNA-Seq). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Borodina to comprise barcoded adaptors as taught by Vaidyanathan. The motivation would be to use an established method for identifying sequencing templates as taught by Vaidyanathan (Para. [0019], commercial synthesis of such 5'- and 3'-modified oligonucleotides can be inefficient and expensive compared to standard (unmodified) oligonucleotides, especially when many oligonucleotides are required for applications such as preparing barcoded libraries for RNA-Seq (Vigneault, 2008); [0058], the methods provide rapid, efficient, and high throughput preparation of oligonucleotides suitable for applications such as barcoding RNA samples for RNA-Seq.).

Claims 15-27 lack an inventive step under PCT Article 33(3) as being obvious over Borodina et al. (hereafter Borodina) in view of Mckernan et al. (hereafter Mckernan) and Vaidyanathan et al. (hereafter Vaidyanathan).

Regarding claim 15, Borodina discloses a method for enriching for target nucleic acid sequences of interest in a sample comprising nucleic acids (pg. 83, Para. 2, detailed protocol for the preparation of an ssRNA-Seq library from mRNA), the method comprising:

- a. fragmenting the nucleic acids, thereby generating nucleic acid fragments (Pg. 83, Para. 3, Day 1: RNA fragmentation.);
- b. appending a first adaptor to the nucleic acid fragments wherein the first adaptor comprises a partial duplex with a short strand and a long strand (Pg. 83 Para. 3, Day 3: adapter ligation; Figure 5.1, showing ligation of Y-adaptors (partial duplex) ligated to 5'ends; Pg. 93, Para. 2, PE genomic adapters; Pg. 94, 2.4.4. Adapter ligation, PE genomic adapter...we first mix the cDNA with the adapters, then mix thoroughly with the buffer, and then add ligase; Pg. 94, 2.4.3. A-Tailing, followed by, 2.4.4. Adapter ligation (A residues are added to the fragments to provide a binding site for an un-paired T residue on the longer (because it has an overhanging T) adapter strand).
- c. denaturing the nucleic acid fragments, thereby creating single-stranded nucleic acid fragments (Pg. 96, 2.4.8. Amplification of the library, 2. Place the reaction in a PCR machine and run the following program: 95 C for 30 s (i.e. denaturation step));
- d. annealing one or more oligonucleotides to the single stranded nucleic acid fragments (Pg. 96, 2.4.8. Amplification of the library, 2. Place the reaction in a PCR machine and run the following program: 95 C for 30 s, followed by cycles of: 95C for 15 s, 65C for 30 s (annealing step), 72 C for 30 s.), whereby each of the one or more oligonucleotides comprise sequence that is complementary to a target nucleic acid sequence of interest present in one or more of the single-stranded nucleic acid fragments (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5'ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTCGGCATTCTGCTGAACCGCTCTTCGATCT-3', # adapter primer 1: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'), extending the one or more oligonucleotides with a polymerase to produce one or more double stranded nucleic acid complexes comprising the target nucleic acid sequences and their complements (Pg. 96, 2.4.8. Amplification of the library, 1. Prepare the amplification reaction mix in a 0.2 ml thin-wall tube: 1X Phusion HF Master Mix (comprises polymerase), 0.4 mM oligos #PE PCR primer 1.1 and #PE PCR primer 2.1. Take 10-30 ml of the library for the reaction. 2. Place the reaction in a PCR machine and run the following program: 95 C for 30 s, followed by cycles of: 95C for 15 s, 65C for 30 s, 72 C for 30 s.). Borodina fails to explicitly disclose wherein a 3' end of the short strand of the partial duplex adaptor comprises a blocking group, and a 5' end of the long strand of the partial duplex adaptor comprises a restriction and/or cleavage site for a nucleic acid modifying enzyme; to synthesize a nucleic acid comprising a first end with a double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme, and a second end with a 3' overhang comprising the short strand of the first adaptor; e. cleaving the double stranded restriction and/or cleavage site with the nucleic acid modifying enzyme, thereby generating a cleavage site; f. ligating a second adaptor to the cleavage site, wherein the second adaptor comprises a duplex with two strands; g. denaturing the one or more double-stranded nucleic acid complexes, thereby generating one or more single stranded nucleic acid fragments comprising the target nucleic acid sequences, a strand from the second adaptor at the first end, and the short strand of the first adaptor at the second end; and h. amplifying the one or more single stranded nucleic acid fragments comprising the one or more target nucleic acid sequences with a first primer comprising sequence complementary to the strand from the second adaptor and a second primer comprising sequence complementary to the short strand of the first adaptor, thereby enriching for the one or more target nucleic acid sequences.

(Claim 15 Continued on next Supplemental Box)

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
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(Continuation of Claim 15 from previous Supplemental Box)

McKernan teaches a method for preparing nucleic acid for analysis (Para. [0005], methods of using a microarray to isolate, purify and/or amplify a target polynucleotide are provided), comprising introduction of a restriction site using an oligonucleotide partial duplex adaptor (Para. [0016], each oligonucleotide primer further comprises at its 5' end a restriction endonuclease recognition site for a distally cleaving restriction endonuclease; [0020], an asymmetrical tail adapter comprising a first ligatable end, and a second end comprising a single-stranded 5' overhang of at least about 8 nucleotides), wherein a 3' end of the short strand of the partial duplex adaptor comprises a blocking group (Para. [0020], wherein the 3' end of the strand that does not comprise the 5' overhang comprises at least one blocking group), and a 5' end of the long strand of the partial duplex adaptor comprises a restriction and/or cleavage site for a nucleic acid modifying enzyme (Para. [0016], each oligonucleotide primer further comprises at its 5' end a restriction endonuclease recognition site for a distally cleaving restriction endonuclease); to synthesize a nucleic acid comprising a first end with a double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme, and a second end with a 3' overhang comprising the short strand of the first adaptor (Paras. [0016]. In one embodiment, the pair of sequencing adapters is a pair of asymmetrical adapters. In a particular embodiment, the pair of asymmetrical adapters comprise a first adapter; [0017], an asymmetrical tail adapter comprising a first ligatable end, and a second end comprising a single-stranded 3' overhang of at least about 8 nucleotides; [0020], a second oligonucleotide adapter selected from the group consisting of (i) an asymmetrical tail adapter comprising a first ligatable end, and a second end comprising a single-stranded 5' overhang of at least about 8 nucleotides, wherein the 3' end of the strand that does not comprise the 5' overhang comprises at least one blocking group; [0098], the first and second asymmetrical adapters further comprise a restriction endonuclease recognition site for a distally cleaving restriction endonuclease, and wherein the nucleic acid sequence of the first and second asymmetrical adapters are not identical; Figure 3, Ligate universal adapter; cut with EcoP151, ligate asymmetric linkers).

e. cleaving the double stranded restriction and/or cleavage site with the nucleic acid modifying enzyme, thereby generating a cleavage site (Para. [0016], the method further comprises cleaving the amplified target polynucleotide with a restriction endonuclease specific for the restriction endonuclease recognition site, which produces a fragment of the amplified target polynucleotide);

f. ligating a second adaptor to the cleavage site, wherein the second adaptor comprises a duplex with two strands (Paras. [0016], the method further comprises joining a pair of sequencing adapters to the fragment of the amplified target polynucleotide, such that one sequencing adapter is joined to each end of the fragment, and each sequencing adapter comprises a primer binding site... the sequencing adapters that are joined to the fragment of the amplified target polynucleotide are a pair of asymmetrical adapters; [0017], an asymmetrical tail adapter comprising a first ligatable end, and a second end comprising a single-stranded 3' overhang of at least about 8 nucleotides);

g. denaturing the one or more double-stranded nucleic acid complexes, thereby generating one or more single stranded nucleic acid fragments comprising the target nucleic acid sequences, a strand from the second adaptor at the first end, and the short strand of the first adaptor at the second end (Para. [0044], Joining the pair of asymmetrical adapters to the ends of the amplified target polynucleotide produces a first adapter-modified target polynucleotide. In a particular embodiment, the method further comprises amplifying the first adapter-modified target polynucleotide to produce an amplified adapter-modified target polynucleotide, [0072], amplification" or an "amplification reaction" refers to methods for amplification of a nucleic acid sequence including polymerase chain reaction (PCR) (denaturing ds nucleic acid to single stranded fragments is an inherent step in PCR amplification); and

h. amplifying the one or more single stranded nucleic acid fragments comprising the one or more target nucleic acid sequences with a first primer comprising sequence complementary to the strand from the second adaptor and a second primer comprising sequence complementary to the short strand of the first adaptor (Para. [0072], Such methods for amplification comprise, e.g., primers that anneal to the nucleic acid sequence to be amplified,), thereby enriching for the one or more target nucleic acid sequences (Paras. [0016], the sequencing adapters that are joined to the fragment of the amplified target polynucleotide are a pair of asymmetrical adapters [0023], each sequencing adapter comprises a primer binding site [0044], the method further comprises amplifying the first adapter-modified target polynucleotide to produce an amplified adapter-modified target polynucleotide; Figure 3, showing amplification after ligation of second adapters). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Borodina to comprise ligation of a second adapter after cleavage of an introduced restriction site as taught by McKernan. The motivation would be to provide sequencing primer binding sites as taught by McKernan (Paras. [0016], the sequencing adapters that are joined to the fragment of the amplified target polynucleotide are a pair of asymmetrical adapters [0023], each sequencing adapter comprises a primer binding site). McKernan fails to explicitly disclose the 3' blocking group remains intact on the fragment of nucleic acid.

Vaidyanathan teaches a method of preparing nucleic acid for sequencing (Para. [0024], The method increases the sensitivity of detection of the bimolecular ligation product by techniques such as RNASeq) comprising ligating an adapter oligonucleotide wherein a 3' end of the short strand of the partial duplex adaptor comprises a blocking group (Para. [0035], a bimolecular ligation reaction between RNA that has a free 3'-hydroxyl end and the 5'-adenylated, 3'-blocked DNA oligonucleotide), and a restriction and/or cleavage site for a nucleic acid modifying enzyme (Para. [0047], In other embodiments, the 5'-adenylated, 3'-blocked DNA donor oligonucleotide contains a restriction endonuclease site adjacent to or in place of the next-generation sequencing adaptor sequence.); to synthesize a nucleic acid comprising a first end with a double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme (Para. [0047], By including a second restriction endonuclease site in a 5'-tagging RNA oligonucleotide, and converting the RNA to a 5'- and 3'-end-tagged, ds cDNA library, the cDNA can be cloned into a suitable vector for further analysis. The second restriction endonuclease site can be the same as, or different from, the first restriction endonuclease site (cleavage of the 5' end leaves 3' blocking group intact)), and a second end with a 3' overhang comprising the short strand of the first adaptor (Figures 1 and 8, showing blocked (X) 3' end of adapted nucleic acid). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Borodina to comprise adaptors with a blocked 3' end and a restriction endonuclease cleavage site as taught by Vaidyanathan. The motivation would be to eliminate ligation or elongation at the blocked 3' terminus, and to provide convenient cloning or ligation sites, as taught by Vaidyanathan (Paras. [0035], the 3'-blocking group of the DNA oligonucleotide is a dideoxynucleotide. Dideoxynucleotides lack hydroxyl groups on both the 2' and 3' positions of the ribose sugar moiety; therefore, they cannot form a 3' to 5' phosphodiester bond; [0036], By including a blocking group on the 3' end of the activated donor DNA oligonucleotide, ligation of one molecule of activated donor DNA oligonucleotide to another is essentially eliminated, thereby avoiding the formation of undesired DNA ligation products; [0047], By including a second restriction endonuclease site in a 5'-tagging RNA oligonucleotide, and converting the RNA to a 5'- and 3'-end-tagged, ds cDNA library, the cDNA can be cloned into a suitable vector for further analysis. The second restriction endonuclease site can be the same as, or different from, the first restriction endonuclease site).

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In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding claim 16, Borodina as modified discloses the method of claim 15. Borodina further discloses an additional step of sequencing the one or more single stranded nucleic acid fragments from step h following amplification (Pg. 83, para. 2, an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing; Figure 5.2, showing sequencing after library amplification).

Regarding claim 17, Borodina as modified discloses the method of claim 15. Borodina further discloses wherein the target nucleic acid sequences of interest comprise genomic DNA, RNA, or cDNA (Pg. 83, Paras. 2, 3, preparation of an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing. The initial step of this part is to generate a cDNA molecule).

Regarding claim 18, Borodina as modified discloses the method of claim 15. Borodina further discloses wherein the target nucleic acid sequences of interest comprise genomic DNA (Pg. 92, Para. 3, based on the original Illumina protocol of library construction from genomic DNA).

Regarding claim 19, Borodina as modified discloses the method of claim 15. Borodina further discloses wherein the target nucleic acid sequences of interest comprise cDNA (Pg. 83, Paras. 2, 3, preparation of an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing. The initial step of this part is to generate a cDNA molecule).

Regarding claim 20, Borodina as modified discloses the method of claim 15. Borodina further discloses wherein the first adaptor and the second adaptor are common to each of the nucleic acid fragments (Pg. 94, 2.4.4. Adapter ligation, Prepare the reaction mix in a 1.5-ml LoBind tube: 10 ml of ds cDNA, PE genomic adapter (the same adapter is ligated to all the fragments), then adapter primers are used to create fragments comprising first (3' segment) and second (priming site for sequencing primer in 5' segment) adapter segments).

Regarding claim 21, Borodina as modified discloses method of claim 15, wherein the first adaptor and the second adaptor are distinct from each other (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5' ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTGGCATTCTGCTGAACCGCTCTCCGATCT-3', # adapter primer 1: 5'-ACACTCTTTCCTACACGACGCTCTCCGATCT-3 (after amplification with adapter primers, the 5' ends have been adapted to comprise a primer binding site suitable for sequencing with Illumina primers); Pg. 83, para. 2, an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing).

Regarding claim 22, Borodina as modified discloses the method of claim 15. Borodina fails to explicitly disclose wherein the first adaptor and/or the second adaptor further comprise barcode sequences.

Vaidyanathan teaches RNA/cDNA libraries (Para. [0024]. The present invention also provides kits for preparing end tagged and dual end-tagged RNA or ds cDNA libraries, suitable for applications such as next-generation sequencing, qPCR, microarray analysis, or cloning) appended with adaptors comprising barcode sequences (Para. [0020], 3' tagging of RNA with barcoded oligonucleotides for RNA-Seq).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Borodina to comprise barcoded adaptors as taught by Vaidyanathan. The motivation would be to use an established method for identifying sequencing templates as taught by Vaidyanathan (Para. [0019], commercial synthesis of such 5' and 3'-modified oligonucleotides can be inefficient and expensive compared to standard (unmodified) oligonucleotides, especially when many oligonucleotides are required for applications such as preparing barcoded libraries for RNA-Seq (Vigneault, 2008); [0058], the methods provide rapid, efficient, and high throughput preparation of oligonucleotides suitable for applications such as barcoding RNA samples for RNA-Seq).

Regarding claim 23, Borodina as modified discloses the method of claim 15. Borodina fails to explicitly disclose wherein the double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme from step e comprises the 5' end of the long strand of the partial duplex of the first adaptor and sequence complementary to the 5' end of the long strand of the partial duplex of the first adaptor generated from extension of the one or more oligonucleotides.

Vaidyanathan teaches wherein the double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme from step e comprises the 5' end of the long strand of the partial duplex of the first adaptor and sequence complementary to the 5' end of the long strand of the partial duplex of the first adaptor generated from extension of the one or more oligonucleotides (Para. [0045]. In other embodiments, the 5'-adenylated, 3'-blocked DNA donor oligonucleotide contains a restriction endonuclease site adjacent to or in place of the next-generation sequencing adaptor sequence. By including a second restriction endonuclease site in a 5'-tagging RNA oligonucleotide, and converting the RNA to a 5'- and 3'-end-tagged, ds cDNA library, the cDNA can be cloned into a suitable vector (the cDNA is generated from extension of the one or more oligonucleotides and comprises sequence complementary to the restriction site at the 5' end of the oligonucleotide)).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Borodina to include wherein the double stranded restriction and/or cleavage site for the nucleic acid modifying enzyme from step e comprises the 5' end of the long strand of the partial duplex of the first adaptor and sequence complementary to the 5' end of the long strand of the partial duplex of the first adaptor generated from extension of the one or more oligonucleotides as taught by Vaidyanathan. The motivation would be to provide for convenient cloning or other manipulations as taught by Vaidyanathan (Para. [0045]. By including a second restriction endonuclease site in a 5'-tagging RNA oligonucleotide, and converting the RNA to a 5'- and 3'-end-tagged, ds cDNA library, the cDNA can be cloned into a suitable vector).

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In case the space in any of the preceding boxes is not sufficient.
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Regarding claim 24, Borodina as modified discloses the method of claim 15. Borodina fails to explicitly disclose wherein the nucleic acid modifying enzyme comprises a restriction enzyme. Vaidyanathan teaches wherein the nucleic acid modifying enzyme comprises a restriction enzyme (Para. [0045]. In other embodiments, the 5'-adenylated, 3'-blocked DNA donor oligonucleotide contains a restriction endonuclease site adjacent to or in place of the next-generation sequencing adaptor sequence. By including a second restriction endonuclease site in a 5'-tagging RNA oligonucleotide, and converting the RNA to a 5'- and 3'-end-tagged, ds cDNA library, the cDNA can be cloned into a suitable vector; Para. [0072] A "restriction site tag" means a tag portion that contains a sequence for the purpose of facilitating cleavage using a restriction endonuclease). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Borodina to comprise wherein the nucleic acid modifying enzyme comprises a restriction enzyme as taught by Vaidyanathan. The motivation would be to provide for convenient cloning or other manipulations as taught by Vaidyanathan (Para. [0045]. By including a second restriction endonuclease site in a 5'-tagging RNA oligonucleotide, and converting the RNA to a 5'- and 3'-end-tagged, ds cDNA library, the cDNA can be cloned into a suitable vector).

Regarding claim 25, Borodina as modified discloses the method of claim 15. Borodina further discloses wherein step b is performed by ligation (Pg. 83, Para. 3, Day 3: adapter ligation; Figure 5.1, showing ligation of Y-adaptors ligated to 5'ends; Pg. 93, Para. 2, PE genomic adapters; Pg. 94, 2.4.4. Adapter ligation, PE genomic adapter...we first mix the cDNA with the adapters, then mix thoroughly with the buffer, and then add ligase).

Regarding claim 26, Borodina as modified discloses the composition comprising the target nucleic sequences of interest enriched by the method of claim 15 (Pg. 96, Para. 2, Load the entire PCR reaction on an agarose gel and excise cDNAs ranging in size between 230 and 270 bp...Proceed to the flowcell preparation, cluster generation, and sequencing reaction on the Illumina Cluster Station).

Regarding claim 27, Borodina as modified discloses the method of claim 15. Borodina further discloses wherein the polymerase is a DNA polymerase (Pg. 96, Prepare the amplification reaction mix in a 0.2 ml thin-wall tube: 1X Phusion HF Master Mix (Phusion HF is a high fidelity thermostable DNA polymerase).

Claim 49 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest:

Regarding claim 49, the prior art of record, individually or in combination, does not teach or fairly suggest the method of claim 41, wherein the first adaptor and the second adaptor are the same.

The following prior art is made of record to support and further define the reason claim 49 meets the criteria set out in PCT Article 33 (2)-(3):

(i) Borodina et al. discloses the method of claim 41 and further discloses an adapter that comprises a sequence for priming amplification, and an additional sequence for priming of sequencing reactions (Figure 5.1, showing oligonucleotides annealed at 3' ends, and additional sequences at 5'ends; Pg. 93, Para. 3, # PE adapter primer 2: 5'-CTCGGCATTCTGCTGAACCGCTCTCCGATCT-3', # adapter primer 1: 5'-ACACTCTTTCCCTACGACGCTCTCCGATCT-3' (after amplification with adapter primers, the 5' ends (i.e. comprising second adapter) have been adapted to comprise a primer binding site suitable for sequencing with Illumina primers); Pg. 83, para. 2, an ssRNA-Seq library from mRNA suitable for Illumina-based sequencing), but fails to explicitly disclose wherein the first adaptor and the second adaptor are the same.

Claims 1-51 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

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Verified and Acknowledged: /ANGELA M. BERTAGNA/

Examiner's Signature

Initials

Title:

COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC
ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY
LIBRARY GENERATION

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
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CONTINUING DATA

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13750768 has PRO of 61591241 01/26/2012

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EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
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			JPO; DERWENT			
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L6	3	I4 and ((ligat\$) near3 (adapter or adaptor or linker)) same ((primer) near3 (tag or tagged or tail or tailed or adapter or adaptor or linker) near3 (target near3 specific\$))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 16:52
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L11	172	((tag or tagged or tail or tailed or adapter or adaptor or linker) near (target or template or gene) near specific near primer) same (amplif\$ or polymerase or pcr) same ((adaptor or adapter or linker or universal or common) near3 primer)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:31
L10	287	((tag or tagged or tail or tailed or adapter or adaptor or linker) near (target or template or gene) near specific near primer)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:31
L9	234	((ligat\$) near5 (adaptor or linker or adapter)) same ((primer or oligonucleotide or oligo) near5 (specific) near5 (target or template or interest) near5 (tail or tailed or tag or tagged or adapter or adaptor or linker)) same ((pcr or polymerase or amplif\$) near5 (adaptor or adapter or linker) near5 primer)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:31
L12	239	((ligat\$) near3 (adapter or adaptor or linker)) same (selectiv\$ near3 (pcr or polymerase or amplif\$) near3 (target or template or genomic))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:32

L13	121	((ligat\$ near5 (adaptor or linker or adapter)) same ((primer or oligonucleotide or oligo) near5 (specific) near5 (target or template or interest) near5 (tail or tailed or tag or tagged or adapter or adaptor or linker)) same ((pcr or polymerase or amplif\$ near5 (adaptor or adapter or linker) near5 primer) same (sequencing or sequenced))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:33
L16	172	((tag or tagged or tail or tailed or adapter or adaptor or linker) near (target or template or gene) near specific near primer) same (amplif\$ or polymerase or pcr) same ((adaptor or adapter or linker or universal or common) near3 primer)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:34
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L14	172	((tag or tagged or tail or tailed or adapter or adaptor or linker) near (target or template or gene) near specific near primer) same (amplif\$ or polymerase or pcr) same ((adaptor or adapter or linker or universal or common) near3 primer)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:34
L17	135	(ligat\$ or ligase or attach\$ or append\$) near8 (adaptor or adapter or linker or linking) near8 ((extended or extension) near3 (product or oligonucleotide)) same (amplif\$ or pcr or (polymerase adj1 chain)) and (primer near5 (tag or tagged or tail or tailed or "not complementary" or (non adj1 complementary))))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:35
L18	103	((ligat\$ near5 (adaptor or linker or adapter)) same ((primer or oligonucleotide or oligo) near5 (specific) near5 (target or template or interest) near5 (tail or tailed or tag or tagged or adapter or adaptor or linker)) same ((pcr or polymerase or	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:36

		amplif\$) near5 (adaptor or adapter or linker) near5 primer) and @ay<="2012"				
L20	81	l19 not l18	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:44
L19	96	((tag or tagged or tail or tailed or adapter or adaptor or linker) near (target or template or gene) near specific near primer) and @ay<="2012"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:44
L21	44	((tag or tagged or tail or tailed or adapter or adaptor or linker) near (target or template or gene) near specific near primer) same (amplif\$ or polymerase or pcr) same ((adaptor or adapter or linker or universal or common) near3 primer) and @ay<="2012"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:46
L24	91	l23 not l19 not l18	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:47
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L22	0	l21 not l19 not l18	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:47
L27	44	((tag or tagged or tail or tailed or adapter or adaptor or linker) near (target or template or gene) near specific near primer) same (amplif\$ or polymerase or pcr) same	US-PGPUB; USPAT; FPRS; EPO;	OR	ON	2020/04/25 17:51

		((adaptor or adapter or linker or universal or common) near3 primer) and @ay<="2012"	JPO; DERWENT			
L26	0	l25 not l24 not l23 not l19 not l18	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:51
L25	28	((ligat\$) near5 (adaptor or linker or adapter)) same ((primer or oligonucleotide or oligo) near5 (specific) near5 (target or template or interest) near5 (tail or tailed or tag or tagged or adapter or adaptor or linker)) same ((pcr or polymerase or amplif\$) near5 (adaptor or adapter or linker) near5 primer) same (sequencing or sequenced) and @ay<="2012"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:51
L30	15	l29 not l24 not l23 not l19 not l18	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:52
L29	40	((ligat\$) near3 (adapter or adaptor or linker)) same ((primer) near3 (tag or tagged or tail or tailed or adapter or adaptor or linker) near3 (target near3 specific\$)) and @ay<="2012"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:52
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L31	44	((tag or tagged or tail or tailed or adapter or adaptor or linker) near (target or template or gene) near specific near primer) same (amplif\$ or polymerase or pcr) same ((adaptor or adapter or linker or universal or common) near3 primer) and @ay<="2012"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:53

L35	34	I34 not I30 not I29 not I24 not I23 not I19 not I18	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:54
L34	48	(ligat\$ or ligase or attach\$ or append\$) near8 (adaptor or adapter or linker or linking) near8 ((extended or extension) near3 (product or oligonucleotide)) same (amplif\$ or pcr or (polymerase adj1 chain)) and (primer near5 (tag or tagged or tail or tailed or "not complementary" or (non adj1 complementary))) and @ay<="2012"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2020/04/25 17:54
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EAST Search History (Interference)

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Substitute for form 1449/PTO			Complete if Known		
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			Filing Date	06-25-2018	
			First Named Inventor	AMORESE; Doug	
			Art Unit	Not Yet Assigned	
			Examiner Name	Not Yet Assigned	
			Attorney Docket Number	25115-755.302	
Sheet	1	of	13		

U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
/A.B/	001	US-4458066	07-03-1984	CARUTHERS; Marvin H [Us] et al.	
	002	US-5525471	06-11-1996	ZENG; Jin	
	003	US-6197501	03-06-2001	CREMER; Thomas et al.	
	004	US-6913884	07-05-2005	STUELPNAGEL; John R. et al.	
	005	US-7056716	06-06-2006	POTTER; Robert Jason et al.	
	006	US-7115400	10-03-2006	ADESSI; Celine et al.	
	007	US-7232656	06-19-2007	BALASUBRAMANIAN; Shankar et al.	
	008	US-7264929	09-04-2007	ROTHBERG; Jonathan M. et al.	
	009	US-7294461	11-13-2007	KURN; Nurith	
	010	US-7351557	04-01-2008	KURN; Nurith	
	011	US-7354717	04-08-2008	KURN; Nurith	
	012	US-7771934	08-10-2010	KURN; Nurith	
	013	US-7771946	08-10-2010	KURN; Nurith	
	014	US-7846666	12-07-2010	KURN; Nurith	
	015	US-7846733	12-07-2010	KURN; Nurith	
	016	US-7867703	01-11-2011	SAMPSON; Jeffrey R. et al.	
	017	US-7939258	05-10-2011	KURN; Nurith et al.	
	018	US-7948015	05-24-2011	ROTHBERG; Jonathan M. et al.	
	019	US-7985565	07-26-2011	KAWASHIMA; Eric H. et al.	
	020	US-8034568	10-11-2011	KURN; Nurith et al.	
	021	US-8071311	12-06-2011	KURN; Nurith	
/A.B/	022	US-8334116	12-18-2012	KURN; Nurith	

Examiner Signature	/ANGELA M. BERTAGNA/	Date Considered	04/21/2020
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ¹ Applicant's unique citation designation number (optional). ² See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. ³ Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). ⁴ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁵ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁶ Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Application Number	16017340
				Filing Date	06-25-2018
				First Named Inventor	AMORESE; Doug
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	2	of	13	Attorney Docket Number	25115-755.302

U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
/A.B./	023	US-8465950	06-18-2013	KURN; Nurith et al.	
	024	US-8492095	07-23-2013	KURN; Nurith	
	025	US-8512956	08-20-2013	KURN; Nurith	
	026	US-8759036	06-24-2014	WANG; Yuker et al.	
	027	US-8852867	10-07-2014	KURN; Nurith et al.	
	028	US-8999677	04-07-2015	SOLDATOV; Aleksey et al.	
	029	US-9175325	11-03-2015	KURN; Nurith et al.	
	030	US-9175336	11-03-2015	SOLDATOV; Aleksey et al.	
	031	US-9181582	11-10-2015	KURN; Nurith	
	032	US-9206418	12-08-2015	ARMOUR; Chris et al.	
	033	US-9650628	05-16-2017	AMORESE; Doug [Us] et al.	
	034	US-9745614	08-29-2017	SCHROEDER; Benjamin G [Us] et al.	
	035	US-9822408	11-21-2017	AMORESE; Doug [Us] et al.	
	036	US-9957549	05-01-2018	ARMOUR; Christopher [Us] et al.	
	037	US-10036012	07-31-2018	AMORESE; Doug [Us] et al.	
	038	US-20060068415	03-30-2006	JONES; Keith W. et al.	
	039	US-20060134633	06-22-2006	CHEN; Yi-Ju et al.	
	040	US-20070141604	06-21-2007	GORMLEY; Niall A. et al.	
	041	US-20080160580	07-03-2008	ADESSI; Celine et al.	
	042	US-20080241831	10-02-2008	FAN; Jian-Bing et al.	
	043	US-20090036663	02-05-2009	KURN; Nurith	
/A.B./	044	US-20090105081	04-23-2009	RODESCH; Matthew et al.	

Examiner Signature	/ANGELA M. BERTAGNA/	Date Considered	04/21/2020
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ¹ Applicant's unique citation designation number (optional). ² See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. ³ Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). ⁴ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁵ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁶ Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Substitute for form 1449/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Application Number	16017340
				Filing Date	06-25-2018
				First Named Inventor	AMORESE; Doug
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	3	of	13	Attorney Docket Number	25115-755.302

U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
/A.B/	045	US-20100081174	04-01-2010	DUNN; John J [Us] et al.	
	046	US-20100105049	04-29-2010	EHRICH; Mathias et al.	
	047	US-20100159559	06-24-2010	KURN; Nurith et al.	
	048	US-20100203597	08-12-2010	CHEN; Liangjing et al.	
	049	US-20100311066	12-09-2010	KURN; Nurith	
	050	US-20110091882	04-21-2011	GRANADOS; Edward N. et al.	
	051	US-20110269194	11-03-2011	MAKAROV; Vladimir	
	052	US-20110294132	12-01-2011	KURN; Nurith	
	053	US-20120003657	01-05-2012	MYLLYKANGAS; Samuel et al.	
	054	US-20120028310	02-02-2012	KURN; Nurith et al.	
	055	US-20120149068	06-14-2012	KURN; Nurith	
	056	US-20120156728	06-21-2012	LI; Bin et al.	
	057	US-20120190587	07-26-2012	KURN; Nurith et al.	
	058	US-20120238738	09-20-2012	HENDRICKSON; Cynthia	
	059	US-20120289426	11-15-2012	ROOS; Fredrik Johansson Henrik Isaksson Magnus Nilsson Mats Ericsson Olle et al.	
	060	US-20140038188	02-06-2014	KURN; Nurith	
	061	US-20140038236	02-06-2014	KURN; Nurith et al.	
	062	US-20140303000	10-09-2014	ARMOUR; Chris et al.	
	063	US-20150225773	08-13-2015	FARMER; Andrew Alan et al.	
/A.B/	064	US-20150284769	10-08-2015	SCHROEDER; Benjamin G. et al.	

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/A.B/	065	US-20160122756	05-05-2016	ARMOUR; Christopher	
↓	066	US-20160153039	06-02-2016	AMORESE; Doug et al.	
	067	US-20160251711	09-01-2016	AMORESE; Doug et al.	
	068	US-20160251712-A1	09-01-2016	AMORESE; Doug et al.	
	069	US-20160265042-A1	09-15-2016	SCHROEDER; Benjamin G. et al.	
	070	US-20160275240	09-22-2016	HUELGA; Stephanie et al.	
↓	071	US-20160275240-A1	09-22-2016	HUELGA; Stephanie et al.	
/A.B/	072	US-20170298345-A1	10-19-2017	AMORESE; Doug et al.	

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		Country Code ³ Number ⁴ Kind Code ⁵ (if known)				
/A.B/	001	EP-2272976-A1	01-12-2011	MAX PLANCK GESELLSCHAFT [DE]		☐
/A.B/	002	EP-2451973-A1 (abstract only)	05-16-2012	MAX PLANCK GESELLSCHAFT [DE]		☐
/A.B/	003	WO-0070039-A1	11-23-2000	UNIV MCGILL [CA], et al.		☐
/A.B/	004	WO-2004092418-A2	10-28-2004	NUGEN TECHNOLOGIES INC [US], et al.		☐

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		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B/	005	WO-2006086668-A2	08-17-2006	EPICT TECHNOLOGIES [US], et al.		☐
/A.B/	006	WO-2007019444-A2	02-15-2007	EUCLID DIAGNOSTICS LLC [US], et al.		☐
/A.B/	007	WO-2009102878-A2	08-20-2009	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	008	WO-2009102896-A2	08-20-2009	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	009	WO-2009117698-A2	09-24-2009	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	010	WO-2010085715-A1	07-29-2010	QUANTA BIOSCIENCES [US], et al.		☐
/A.B/	011	WO-2011019964-A1	02-17-2011	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	012	WO-2011053987-A1	05-05-2011	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	013	WO-2011151777-A1	12-08-2011	CONSIGLIO NAZIONALE RICERCHE [IT], et al.		☐
/A.B/	014	WO-2012013932-A1	02-02-2012	UNIV ST ANDREWS [GB], et al.		☐

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		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B/	015	WO-2013059740-A1	04-25-2013	FOUNDATION MEDICINE INC [US]		☐
/A.B/	016	WO-2013112923-A1	08-01-2013	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	017	WO-2014144092-A1	09-18-2014	NUGEN TECHNOLOGIES INC [US]		☐
/A.B/	018	WO-2014150931-A1	09-25-2014	NUGEN TECHNOLOGIES INC [US]		☐
/A.B/	019	WO-2015122967-A1	08-20-2015	CLONTECH LAB INC [US]		☐
/A.B/	020	WO-2015131107-A1	09-03-2015	NUGEN TECHNOLOGIES INC [US]		☐
/A.B/	021	WO-2016033251-A2	03-03-2016	NUGEN TECHNOLOGIES INC [US]		☐
/A.B/	022	WO-2016100955-A2	06-23-2016	IDENTIFYGENOMI CS LLC [US]		☐

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NON-PATENT LITERATURE DOCUMENTS			
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/A.B./	001	BALDWIN et al. Multilocus sequence typing of Cronobacter sakazakii and Cronobacter malonaticus reveals stable clonal structures with clinical significance which do not correlate with biotypes. BMC Microbiology 9:223. 9 pages. (October 23, 2009). DOI: https://doi.org/10.1186/1471-2180-9-223 .	☐
/A.B./	002	BEAUCAGE et al. Deoxynucleoside Phosphoramidites-A New Class of Key Intermediates for Deoxypolynucleotide Synthesis. Tetrahedron Letters. 1981;22(20):1859-1862.	☐
/A.B./	003	BigDye Terminator v3.1 Cycle Sequencing Kit. Applied Biosystems, pp. 1-72 (2002).	☐
/A.B./	004	BODI, et al. Comparison of Commercially Available Target Enrichment Methods for Next-Generation Sequencing. J Biomol Tech. 2013 Jul; 24(2): 73-86.	☐
/A.B./	005	BROWN, et al. Chemical synthesis and cloning of a tyrosine tRNA gene. Methods Enzymol. 1979;68:109-51.	☐
	006	KURN, et al., United States Patent Application No. US13/980987, filed on 07-22-2013.	☐
/A.B./	007	AMORESE, et al., United States Patent Application No. US14/836936, filed on 08-26-2015.	☐
/A.B./	008	European search report and opinion dated 01/29/2016 for EP Application No. 13806978.	☐
/A.B./	009	FROUSSARD. A random-PCR method (rPCR) to construct whole cDNA library from low amounts of RNA. Nucleic Acids Res. 1992 Jun 11;20(11):2900.	☐
/A.B./	010	GUDMUNDSSON, et al. Genome-wide association and replication studies identify four variants associated with prostate cancer susceptibility. Nat Genet. 2009 Oct;41(10):1122-6. doi: 10.1038/ng.448. Epub 2009 Sep 20.	☐

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/A.B./	011	HUANG et al., The behavior of 5-hydroxymethylcytosine in Bisulfite sequencing. PLOS one, January 2010; 5(1):e8888. 9 Pages.	☐
/A.B./	012	International search report and written opinion dated 02/05/2016 for PCT/US2015/047053.	☐
/A.B./	013	JANG, et al., Spectra of BRCA1 and BRCA2 mutations in Korean patients with breast cancer: the importance of whole-gene sequencing. Journal of Human Genetics. Vol. 57. Pages 212-215. January 5, 2012.	☐
/A.B./	014	KURN, Nurith, United States Patent Application No. 14/877075, filed 10/07/2015.	☐
/A.B./	015	LEVESQUE-SERGERIE, et al. Detection limits of several commercial reverse transcriptase enzymes: impact on the low- and high-abundance transcript levels assessed by quantitative RT-PCR. BMC Mol Biol. 2007 Oct 22;8:93.	☐
/A.B./	016	MULLIGAN et al., Differential binding of Escherichia coli McrA protein to DNA sequences that contain the dinucleotide m5CpG. Nucleic Acids Research, 2010; 38(6):1997-2005.	☐
/A.B./	017	Multi-Site Study Validates End-to-End RNA-Seq Sample Prep Workflow for Human Whole Blood Clinical Samples. Nugen Technologies Press Release. San Carlos. July 15, 2015. 3 pages. http://www.businesswire.com/news/home/20150715005371/en#.VaZOCPIWLKE .	☐
/A.B./	018	NARANG, et al. Improved phosphotriester method for the synthesis of gene fragments. Methods Enzymol. 1979;68:90-8.	☐
/A.B./	019	Notice of allowance dated 01/05/2017 for US Application No. 13/750,768.	☐
/A.B./	020	Notice of Allowance dated 04/17/2017 for US Application No. 14/634,326.	☐
/A.B./	021	Notice of Allowance dated 06/21/2017 for US Application No. 14/634,326.	☐

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/A.B./	022	Notice of Allowance dated 07/25/2017 for US Application No. 14/778,564. 9 Pages.	☐
/A.B./	023	Notice of Allowance dated 12/26/2017 for U.S. Patent Application No. 14/390,012. 7 Pages.	☐
/A.B./	024	NuGEN Launches Enhanced Target Enrichment Technology Allowing More Comprehensive and Efficient NGS Analysis of Cancer Samples. San Carlos, CA. August 03, 2015. 2 pages.	☐
/A.B./	025	NuGEN scientists screen 400+ genes for fusion events in single assay; detect new fusions and those previously associated with cancers. Nugen Technologies. San Carlos. July 1, 2015.	☐
/A.B./	026	Office action dated 02/05/2014 for US Application No. 13/750,768.	☐
/A.B./	027	Office action dated 04/04/2016 for US Application No. 14/995,882.	☐
/A.B./	028	Office action dated 04/07/2016 for US Application No. 14/390,012.	☐
/A.B./	029	Office action dated 06/02/2016 for US Application No. 13/750,768.	☐
/A.B./	030	Office action dated 07/15/2015 for US Application No. 13/750,768.	☐
/A.B./	031	Office Action dated 08/07/2017 for US Application No. 14/920,254. 9 Pages.	☐
/A.B./	032	Office Action dated 08/18/2017 for U.S. Patent Application No. 15/471,785.	☐
/A.B./	033	Office Action dated 08/22/2017 for U.S. Patent Application No. 14/836,936. 20 Pages.	☐
/A.B./	034	Office Action dated 08/30/2017 for U.S. Patent Application No. 14/990,339. 9 Pages.	☐
/A.B./	035	Office action dated 09/25/2014 for US Application No. 13/750,768.	☐

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Sheet	10	of	13	Attorney Docket Number	25115-755.302

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Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
/A.B./	036	Office action dated 10/30/2015 for US Application No. 13/750,768.	☐
/A.B./	037	Office Action dated 12/15/2017 for U.S. Patent Application No. 14/991,340, 14 Pages.	☐
/A.B./	038	Office Action dated 7/19/2017 for US Application No. 14/390,012. 12 Pages.	☐
/A.B./	039	OUT, et al. Deep sequencing to reveal new variants in pooled DNA samples. Hum Mutat. 2009 Dec; 30(12): 1703-12. doi: 10.1002/humu.21122.	☐
/A.B./	040	Ovation® Target Enrichment System. User guide. Nugen. 2016. 45 pages.	☐
/A.B./	041	SCOLNICK, et al. An Efficient Method for Identifying Gene Fusions by Targeted RNA Sequencing from Fresh Frozen and FFPE Samples. PLoS ONE 10(7): e0128916. doi:10.1371/journal.pone.0128916.	☐
/A.B./	042	U.S. Serial No. 14/990,339 Office Action dated August 29, 2018.	☐
/A.B./	043	U.S. Serial No. 14/991,340 Office Action dated September 28, 2018.	☐
/A.B./	044	U.S. Serial No. 14/990,339 Office Action dated January 3, 2018.	☐
/A.B./	045	TURNER, et al. Massively parallel exon capture and library-free resequencing across 16 genomes. Nat Methods. 2009 May; 6(5): 315-6. doi: 10.1038/nmeth.f.248. Epub 2009 Apr 6.	☐
/A.B./	046	U.S. 15/471,785 Notice of Allowance dated April 10, 2018.	☐
/A.B./	047	U.S. 15/471,785 Notice of Allowance dated June 21, 2018.	☐
/A.B./	048	U.S. Serial 14/920,254 Office Action dated March 15, 2018, 13 pages.	☐

Examiner Signature	/ANGELA M. BERTAGNA/	Date Considered	04/21/2020
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Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Complete if Known			
		Application Number	16017340		
		Filing Date	06-25-2018		
		First Named Inventor	AMORESE; Doug		
		Art Unit	Not Yet Assigned		
		Examiner Name	Not Yet Assigned		
Sheet	11	of	13	Attorney Docket Number	25115-755.302

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
/A.B./	049	VATER, et al. Short bioactive Spiegelmers to migraine-associated calcitonin gene-related peptide rapidly identified by a novel approach: tailored-SELEX. Nucleic Acids Res. 2003 Nov 1;31(21):e130.	☐
/A.B./	050	VOELKERDING, et al. Next generation sequencing: from basic research to diagnostics. Clin. Chem. 2009; 55(4):641-658.	☐
/A.B./	051	WATSON, et al. Cloning and assembly of PCR products using modified primers and DNA repair enzymes. Biotechniques. 1997 Nov;23(5):858-62, 864.	☐
/A.B./	052	WEBER et al. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat. Genet. 37:853-862 (2005).	☐
/A.B./	053	ZHANG, et al. Copy number variation in human health, disease, and evolution. Annu Rev Genomics Hum Genet. 2009;10:451-81. doi: 10.1146/annurev.genom.9.081307.164217.	☐
/A.B./	054	ZHONG, et al. High-throughput illumina strand-specific RNA sequencing library preparation. Cold Spring Harb. Protoc.; 2011; 940-949. doi:10.1101/pdb.prot5652.	☐

Examiner Signature	/ANGELA M. BERTAGNA/	Date Considered	04/21/2020
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Application Number	16017340
		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
Sheet	12	of	13

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

- ☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

- ☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

- ☐ See attached certification statement.

- ☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

- ☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jeffrey Seidel/	Date (YYYY-MM-DD)	2018-12-04
Name/Print	Jeffrey Seidel	Registration Number	61,063

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Substitute for form 1449/PTO			Complete if Known		
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)			Application Number	16017340	
			Filing Date	06-25-2018	
			First Named Inventor	AMORESE; Doug	
			Art Unit	Not Yet Assigned	
			Examiner Name	Not Yet Assigned	
			Attorney Docket Number	25115-755.302	
Sheet	1	of	13		

U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
/A.B/	001	US-4458066	07-03-1984	CARUTHERS; Marvin H [Us] et al.	
	002	US-5525471	06-11-1996	ZENG; Jin	
	003	US-6197501	03-06-2001	CREMER; Thomas et al.	
	004	US-6913884	07-05-2005	STUELPNAGEL; John R. et al.	
	005	US-7056716	06-06-2006	POTTER; Robert Jason et al.	
	006	US-7115400	10-03-2006	ADESSI; Celine et al.	
	007	US-7232656	06-19-2007	BALASUBRAMANIAN; Shankar et al.	
	008	US-7264929	09-04-2007	ROTHBERG; Jonathan M. et al.	
	009	US-7294461	11-13-2007	KURN; Nurith	
	010	US-7351557	04-01-2008	KURN; Nurith	
	011	US-7354717	04-08-2008	KURN; Nurith	
	012	US-7771934	08-10-2010	KURN; Nurith	
	013	US-7771946	08-10-2010	KURN; Nurith	
	014	US-7846666	12-07-2010	KURN; Nurith	
	015	US-7846733	12-07-2010	KURN; Nurith	
	016	US-7867703	01-11-2011	SAMPSON; Jeffrey R. et al.	
	017	US-7939258	05-10-2011	KURN; Nurith et al.	
	018	US-7948015	05-24-2011	ROTHBERG; Jonathan M. et al.	
	019	US-7985565	07-26-2011	KAWASHIMA; Eric H. et al.	
	020	US-8034568	10-11-2011	KURN; Nurith et al.	
	021	US-8071311	12-06-2011	KURN; Nurith	
/A.B/	022	US-8334116	12-18-2012	KURN; Nurith	

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/A.B./	023	US-8465950	06-18-2013	KURN; Nurith et al.	
	024	US-8492095	07-23-2013	KURN; Nurith	
	025	US-8512956	08-20-2013	KURN; Nurith	
	026	US-8759036	06-24-2014	WANG; Yuker et al.	
	027	US-8852867	10-07-2014	KURN; Nurith et al.	
	028	US-8999677	04-07-2015	SOLDATOV; Aleksey et al.	
	029	US-9175325	11-03-2015	KURN; Nurith et al.	
	030	US-9175336	11-03-2015	SOLDATOV; Aleksey et al.	
	031	US-9181582	11-10-2015	KURN; Nurith	
	032	US-9206418	12-08-2015	ARMOUR; Chris et al.	
	033	US-9650628	05-16-2017	AMORESE; Doug [Us] et al.	
	034	US-9745614	08-29-2017	SCHROEDER; Benjamin G [Us] et al.	
	035	US-9822408	11-21-2017	AMORESE; Doug [Us] et al.	
	036	US-9957549	05-01-2018	ARMOUR; Christopher [Us] et al.	
	037	US-10036012	07-31-2018	AMORESE; Doug [Us] et al.	
	038	US-20060068415	03-30-2006	JONES; Keith W. et al.	
	039	US-20060134633	06-22-2006	CHEN; Yi-Ju et al.	
	040	US-20070141604	06-21-2007	GORMLEY; Niall A. et al.	
	041	US-20080160580	07-03-2008	ADESSI; Celine et al.	
	042	US-20080241831	10-02-2008	FAN; Jian-Bing et al.	
	043	US-20090036663	02-05-2009	KURN; Nurith	
/A.B./	044	US-20090105081	04-23-2009	RODESCH; Matthew et al.	

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/A.B/	045	US-20100081174	04-01-2010	DUNN; John J [Us] et al.	
	046	US-20100105049	04-29-2010	EHRICH; Mathias et al.	
	047	US-20100159559	06-24-2010	KURN; Nurith et al.	
	048	US-20100203597	08-12-2010	CHEN; Liangjing et al.	
	049	US-20100311066	12-09-2010	KURN; Nurith	
	050	US-20110091882	04-21-2011	GRANADOS; Edward N. et al.	
	051	US-20110269194	11-03-2011	MAKAROV; Vladimir	
	052	US-20110294132	12-01-2011	KURN; Nurith	
	053	US-20120003657	01-05-2012	MYLLYKANGAS; Samuel et al.	
	054	US-20120028310	02-02-2012	KURN; Nurith et al.	
	055	US-20120149068	06-14-2012	KURN; Nurith	
	056	US-20120156728	06-21-2012	LI; Bin et al.	
	057	US-20120190587	07-26-2012	KURN; Nurith et al.	
	058	US-20120238738	09-20-2012	HENDRICKSON; Cynthia	
	059	US-20120289426	11-15-2012	ROOS; Fredrik Johansson Henrik Isaksson Magnus Nilsson Mats Ericsson Olle et al.	
	060	US-20140038188	02-06-2014	KURN; Nurith	
	061	US-20140038236	02-06-2014	KURN; Nurith et al.	
	062	US-20140303000	10-09-2014	ARMOUR; Chris et al.	
	063	US-20150225773	08-13-2015	FARMER; Andrew Alan et al.	
/A.B/	064	US-20150284769	10-08-2015	SCHROEDER; Benjamin G. et al.	

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/A.B/	065	US-20160122756	05-05-2016	ARMOUR; Christopher	
↓	066	US-20160153039	06-02-2016	AMORESE; Doug et al.	
	067	US-20160251711	09-01-2016	AMORESE; Doug et al.	
	068	US-20160251712-A1	09-01-2016	AMORESE; Doug et al.	
	069	US-20160265042-A1	09-15-2016	SCHROEDER; Benjamin G. et al.	
	070	US-20160275240	09-22-2016	HUELGA; Stephanie et al.	
↓	071	US-20160275240-A1	09-22-2016	HUELGA; Stephanie et al.	
/A.B/	072	US-20170298345-A1	10-19-2017	AMORESE; Doug et al.	

FOREIGN PATENT DOCUMENTS						
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		Country Code ³ Number ⁴ Kind Code ⁵ (if known)				
/A.B/	001	EP-2272976-A1	01-12-2011	MAX PLANCK GESELLSCHAFT [DE]		☐
/A.B/	002	EP-2451973-A1 (abstract only)	05-16-2012	MAX PLANCK GESELLSCHAFT [DE]		☐
/A.B/	003	WO-0070039-A1	11-23-2000	UNIV MCGILL [CA], et al.		☐
/A.B/	004	WO-2004092418-A2	10-28-2004	NUGEN TECHNOLOGIES INC [US], et al.		☐

Examiner Signature	/ANGELA M. BERTAGNA/	Date Considered	04/21/2020
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Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	16017340
				Filing Date	06-25-2018
				First Named Inventor	AMORESE, Doug
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	5	of	13	Attorney Docket Number	25115-755.302

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No. ¹	Foreign Patent Document	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T ⁶
		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B/	005	WO-2006086668-A2	08-17-2006	EPICT TECHNOLOGIES [US], et al.		☐
/A.B/	006	WO-2007019444-A2	02-15-2007	EUCLID DIAGNOSTICS LLC [US], et al.		☐
/A.B/	007	WO-2009102878-A2	08-20-2009	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	008	WO-2009102896-A2	08-20-2009	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	009	WO-2009117698-A2	09-24-2009	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	010	WO-2010085715-A1	07-29-2010	QUANTA BIOSCIENCES [US], et al.		☐
/A.B/	011	WO-2011019964-A1	02-17-2011	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	012	WO-2011053987-A1	05-05-2011	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	013	WO-2011151777-A1	12-08-2011	CONSIGLIO NAZIONALE RICERCHE [IT], et al.		☐
/A.B/	014	WO-2012013932-A1	02-02-2012	UNIV ST ANDREWS [GB], et al.		☐

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		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B./	015	WO-2013059740-A1	04-25-2013	FOUNDATION MEDICINE INC [US]		☐
/A.B./	016	WO-2013112923-A1	08-01-2013	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B./	017	WO-2014144092-A1	09-18-2014	NUGEN TECHNOLOGIES INC [US]		☐
/A.B./	018	WO-2014150931-A1	09-25-2014	NUGEN TECHNOLOGIES INC [US]		☐
/A.B./	019	WO-2015122967-A1	08-20-2015	CLONTECH LAB INC [US]		☐
/A.B./	020	WO-2015131107-A1	09-03-2015	NUGEN TECHNOLOGIES INC [US]		☐
/A.B./	021	WO-2016033251-A2	03-03-2016	NUGEN TECHNOLOGIES INC [US]		☐
/A.B./	022	WO-2016100955-A2	06-23-2016	IDENTIFYGENOMI CS LLC [US]		☐

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/A.B./	001	BALDWIN et al. Multilocus sequence typing of Cronobacter sakazakii and Cronobacter malonaticus reveals stable clonal structures with clinical significance which do not correlate with biotypes. BMC Microbiology 9:223. 9 pages. (October 23, 2009). DOI: https://doi.org/10.1186/1471-2180-9-223 .	☐
/A.B./	002	BEAUCAGE et al. Deoxynucleoside Phosphoramidites-A New Class of Key Intermediates for Deoxypolynucleotide Synthesis. Tetrahedron Letters. 1981;22(20):1859-1862.	☐
/A.B./	003	BigDye Terminator v3.1 Cycle Sequencing Kit. Applied Biosystems, pp. 1-72 (2002).	☐
/A.B./	004	BODI, et al. Comparison of Commercially Available Target Enrichment Methods for Next-Generation Sequencing. J Biomol Tech. 2013 Jul; 24(2): 73-86.	☐
/A.B./	005	BROWN, et al. Chemical synthesis and cloning of a tyrosine tRNA gene. Methods Enzymol. 1979;68:109-51.	☐
	006	KURN, et al., United States Patent Application No. US13/980987, filed on 07-22-2013.	☐
/A.B./	007	AMORESE, et al., United States Patent Application No. US14/836936, filed on 08-26-2015.	☐
/A.B./	008	European search report and opinion dated 01/29/2016 for EP Application No. 13806978.	☐
/A.B./	009	FROUSSARD. A random-PCR method (rPCR) to construct whole cDNA library from low amounts of RNA. Nucleic Acids Res. 1992 Jun 11;20(11):2900.	☐
/A.B./	010	GUDMUNDSSON, et al. Genome-wide association and replication studies identify four variants associated with prostate cancer susceptibility. Nat Genet. 2009 Oct;41(10):1122-6. doi: 10.1038/ng.448. Epub 2009 Sep 20.	☐

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/A.B./	011	HUANG et al., The behavior of 5-hydroxymethylcytosine in Bisulfite sequencing. PLOS one, January 2010; 5(1):e8888. 9 Pages.	☐
/A.B./	012	International search report and written opinion dated 02/05/2016 for PCT/US2015/047053.	☐
/A.B./	013	JANG, et al., Spectra of BRCA1 and BRCA2 mutations in Korean patients with breast cancer: the importance of whole-gene sequencing. Journal of Human Genetics. Vol. 57. Pages 212-215. January 5, 2012.	☐
	014	KURN, North, United States Patent Application No. 14/877075, filed 10/07/2015.	☐
/A.B./	015	LEVESQUE-SERGERIE, et al. Detection limits of several commercial reverse transcriptase enzymes: impact on the low- and high-abundance transcript levels assessed by quantitative RT-PCR. BMC Mol Biol. 2007 Oct 22;8:93.	☐
/A.B./	016	MULLIGAN et al., Differential binding of Escherichia coli McrA protein to DNA sequences that contain the dinucleotide m5CpG. Nucleic Acids Research, 2010; 38(6):1997-2005.	☐
/A.B./	017	Multi-Site Study Validates End-to-End RNA-Seq Sample Prep Workflow for Human Whole Blood Clinical Samples. Nugen Technologies Press Release. San Carlos. July 15, 2015. 3 pages. http://www.businesswire.com/news/home/20150715005371/en#.VaZOCPIWLKE .	☐
/A.B./	018	NARANG, et al. Improved phosphotriester method for the synthesis of gene fragments. Methods Enzymol. 1979;68:90-8.	☐
/A.B./	019	Notice of allowance dated 01/05/2017 for US Application No. 13/750,768.	☐
/A.B./	020	Notice of Allowance dated 04/17/2017 for US Application No. 14/634,326.	☐
/A.B./	021	Notice of Allowance dated 06/21/2017 for US Application No. 14/634,326.	☐

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/A.B./	022	Notice of Allowance dated 07/25/2017 for US Application No. 14/778,564. 9 Pages.	☐
/A.B./	023	Notice of Allowance dated 12/26/2017 for U.S. Patent Application No. 14/390,012. 7 Pages.	☐
/A.B./	024	NuGEN Launches Enhanced Target Enrichment Technology Allowing More Comprehensive and Efficient NGS Analysis of Cancer Samples. San Carlos, CA. August 03, 2015. 2 pages.	☐
/A.B./	025	NuGEN scientists screen 400+ genes for fusion events in single assay; detect new fusions and those previously associated with cancers. Nugen Technologies. San Carlos. July 1, 2015.	☐
/A.B./	026	Office action dated 02/05/2014 for US Application No. 13/750,768.	☐
/A.B./	027	Office action dated 04/04/2016 for US Application No. 14/995,882.	☐
/A.B./	028	Office action dated 04/07/2016 for US Application No. 14/390,012.	☐
/A.B./	029	Office action dated 06/02/2016 for US Application No. 13/750,768.	☐
/A.B./	030	Office action dated 07/15/2015 for US Application No. 13/750,768.	☐
/A.B./	031	Office Action dated 08/07/2017 for US Application No. 14/920,254. 9 Pages.	☐
/A.B./	032	Office Action dated 08/18/2017 for U.S. Patent Application No. 15/471,785.	☐
/A.B./	033	Office Action dated 08/22/2017 for U.S. Patent Application No. 14/836,936. 20 Pages.	☐
/A.B./	034	Office Action dated 08/30/2017 for U.S. Patent Application No. 14/990,339. 9 Pages.	☐
/A.B./	035	Office action dated 09/25/2014 for US Application No. 13/750,768.	☐

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/A.B./	036	Office action dated 10/30/2015 for US Application No. 13/750,768.	☐
/A.B./	037	Office Action dated 12/15/2017 for U.S. Patent Application No. 14/991,340, 14 Pages.	☐
/A.B./	038	Office Action dated 7/19/2017 for US Application No. 14/390,012. 12 Pages.	☐
/A.B./	039	OUT, et al. Deep sequencing to reveal new variants in pooled DNA samples. Hum Mutat. 2009 Dec; 30(12): 1703-12. doi: 10.1002/humu.21122.	☐
/A.B./	040	Ovation® Target Enrichment System. User guide. Nugen. 2016. 45 pages.	☐
/A.B./	041	SCOLNICK, et al. An Efficient Method for Identifying Gene Fusions by Targeted RNA Sequencing from Fresh Frozen and FFPE Samples. PLoS ONE 10(7): e0128916. doi:10.1371/journal.pone.0128916.	☐
/A.B./	042	U.S. Serial No. 14/990,339 Office Action dated August 29, 2018.	☐
/A.B./	043	U.S. Serial No. 14/991,340 Office Action dated September 28, 2018.	☐
/A.B./	044	U.S. Serial No. 14/990,339 Office Action dated January 3, 2018.	☐
/A.B./	045	TURNER, et al. Massively parallel exon capture and library-free resequencing across 16 genomes. Nat Methods. 2009 May; 6(5): 315-6. doi: 10.1038/nmeth.f.248. Epub 2009 Apr 6.	☐
/A.B./	046	U.S. 15/471,785 Notice of Allowance dated April 10, 2018.	☐
/A.B./	047	U.S. 15/471,785 Notice of Allowance dated June 21, 2018.	☐
/A.B./	048	U.S. Serial 14/920,254 Office Action dated March 15, 2018, 13 pages.	☐

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/A.B./	049	VATER, et al. Short bioactive Spiegelmers to migraine-associated calcitonin gene-related peptide rapidly identified by a novel approach: tailored-SELEX. Nucleic Acids Res. 2003 Nov 1;31(21):e130.	☐
/A.B./	050	VOELKERDING, et al. Next generation sequencing: from basic research to diagnostics. Clin. Chem. 2009; 55(4):641-658.	☐
/A.B./	051	WATSON, et al. Cloning and assembly of PCR products using modified primers and DNA repair enzymes. Biotechniques. 1997 Nov;23(5):858-62, 864.	☐
/A.B./	052	WEBER et al. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat. Genet. 37:853-862 (2005).	☐
/A.B./	053	ZHANG, et al. Copy number variation in human health, disease, and evolution. Annu Rev Genomics Hum Genet. 2009;10:451-81. doi: 10.1146/annurev.genom.9.081307.164217.	☐
/A.B./	054	ZHONG, et al. High-throughput illumina strand-specific RNA sequencing library preparation. Cold Spring Harb. Protoc.; 2011; 940-949. doi:10.1101/pdb.prot5652.	☐

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Sheet	12	of	13

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

- ☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

- ☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

- ☐ See attached certification statement.
- ☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.
- ☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jeffrey Seidel/	Date (YYYY-MM-DD)	2018-12-04
Name/Print	Jeffrey Seidel	Registration Number	61,063

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/017,340	06/25/2018	Doug AMORESE	NUGN-037/04US 34079/440	2635
21710	7590	04/30/2020	EXAMINER	
BROWN RUDNICK LLP ONE FINANCIAL CENTER BOSTON, MA 02111			BERTAGNA, ANGELA MARIE	
			ART UNIT	PAPER NUMBER
			1637	
			NOTIFICATION DATE	DELIVERY MODE
			04/30/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ip@brownrudnick.com
usactions@brownrudnick.com

Office Action Summary

Application No.
16/017,340

Applicant(s)
AMORESE et al.

Examiner
Angela Bertagna

Art Unit
1637

AIA (FITF) Status
No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on 11/30/18.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.

2a) ☐ This action is **FINAL**.

2b) ☒ This action is non-final.

3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.

4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☒ Claim(s) 51-70 is/are pending in the application.

5a) Of the above claim(s) ____ is/are withdrawn from consideration.

6) ☐ Claim(s) ____ is/are allowed.

7) ☒ Claim(s) 51-70 is/are rejected.

8) ☒ Claim(s) 51,56-58,61 and 64 is/are objected to.

9) ☐ Claim(s) ____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see

http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10) ☒ The specification is objected to by the Examiner.

11) ☒ The drawing(s) filed on 6/25/18 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☐ All b) ☐ Some** c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. ____.

3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

3) ☐ Interview Summary (PTO-413)

Paper No(s)/Mail Date ____.

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)

4) ☐ Other: ____.

Paper No(s)/Mail Date ____.

DETAILED CORRESPONDENCE

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejections set forth below will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

Preliminary Amendment

2. The preliminary amendment filed on November 30, 2018 has been entered. Claims 51-70 are pending.

It is noted that declaration evidence concerning a method quite similar to the method recited in the instant claims was presented in the parent and grandparent applications. Since such evidence does not automatically become part of the instant application, Applicant is advised to submit a copy of the declaration evidence and any supporting arguments if consideration of said evidence is desired.

Information Disclosure Statement

3. The Information Disclosure Statement (IDS) containing 61 pages filed on December 4, 2018 fails to comply with 37 CFR 1.98(a)(2), which requires a legible copy of each cited foreign patent document; each non-patent literature publication or that portion which caused it to be listed; and all other information or that portion which caused it to be listed. The transmittal letter

accompanying this IDS states that the copies may be found in the grandparent application (13/750,768), but copies of the citations that have been lined through were not found in the grandparent application.

Applicant is advised that the date of any re-submission of any item of information contained in this IDS or the submission of any missing element(s) will be the date of submission for purposes of determining compliance with the requirements based on the time of filing the statement, including all certification requirements for statements under 37 CFR 1.97(e). See MPEP § 609.05(a).

Specification

4. The specification is objected to because the continuity information in the first paragraph should be updated to indicate that prior-filed Application Serial No. 15/471,785 has issued as U.S. Patent 10,036,012.

Claim Objections

5. Claim 51 is objected to because of the following informalities. Amending step (c) to replace “, thereby generating” in line 4 of the step with “by generating” is suggested. Also, replacing “an enriched nucleic acid” in the last line of step (c) with “the enriched nucleic acid is suggested” to maintain consistency within the step. As well, amending step (a) to replace “the nucleic acid fragment” in line 3 of the step with “the nucleic acid fragment to which the one or more oligonucleotides are annealed” or “the nucleic acid fragment comprising the nucleic acid sequence of interest” is suggested to improve clarity.

Claim 56 is objected to because “comprise” in line 1 should be replaced with “comprises.” As well, deleting “each of” in line 2 is suggested.

Claim 57 is objected to because the word “the” should be inserted before the word “second” in line 2.

Claim 58 is objected to because “comprise” in line 2 should be replaced with “comprise(s)” to account for the “and” option as well as the “or” option.

Claim 61 is objected to because the word “the” should be inserted before “use” in line 1.

Claim 64 is objected to because of the following informalities. Deleting “of each of the plurality of nucleic acid fragments” is suggested since it is redundant.

Claim Rejections - 35 USC § 112

6. The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 59 and 66 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Claim 59 is indefinite because it appears to be missing at least one element or step. The claim depends from claim 51 and recites “further comprising denaturing double-stranded nucleic acid fragments prior to step a), thereby generating the nucleic acid fragment comprising the first adaptor sequence.” It is clear that this claim requires performance of a denaturation step before the annealing of step (a), but it is not clear how a denaturation step can generate an adaptor-

containing nucleic acid fragment. It seems that an additional step (e.g., adaptor ligation or polymerase-mediated adaptor addition) is needed to generate an adaptor-containing nucleic acid fragment. The claim would be clear and definite if the “thereby” clause was deleted.

Claim 66 is indefinite because the metes and bounds of “appended to” are unclear. The specification states that ligation is encompassed by the term, it is not clear as to what else is encompassed by “appended to.” Neither the specification nor the claims gives any additional description as to what is encompassed by this language. And, more specifically, it is unclear whether, for example, “appended to” also encompasses partial duplex adaptors that are merely hybridized to the nucleic acid fragments.

Claim Rejections - 35 USC § 103

7. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

8. This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

9. Claims 51, 52, 54-61, and 63-70 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jones et al. (US 2005/0142577 A1; IDS reference) in view of Tucker et al. (The American Journal of Human Genetics 2009; **85**: 142-154; IDS reference).

Regarding claims 51, 56, 57, 60, and 64-67, Jones teaches a method for enriching a nucleic acid sequence of interest in a sample comprising nucleic acids that comprises the following steps (see, for example, Figures 3-4 and paragraphs 86-90, 96, 99, and 100):

(a) fragmenting nucleic acids in the sample to generate fragments comprising the sequence of interest;

(b) ligating a partial duplex adaptor to each end of the nucleic acid fragments, thereby providing a plurality of fragments having a common first adapter sequence ligated at a 5' end;

(c) annealing one or more oligonucleotides in solution to the nucleic acid sequence of interest in the fragments, wherein said oligonucleotides comprise a 3' portion with at least ten bases that are complementary to the nucleic acid sequence of interest and a 5' portion comprising a common second adaptor sequence that is not complementary to the nucleic acid sequence of interest;

(d) extending said annealed oligonucleotides using a DNA polymerase, thereby obtaining extension products comprising sequence complementary to the ligated first adaptor, sequence complementary to the nucleic acid of interest, and the second adaptor;

(e) amplifying the extension products using a primer that anneals to the complement of the first adaptor sequence and a primer that anneals to the complement of the second adaptor sequence to enrich for the nucleic acid sequence of interest; and

(f) sequencing the enriched nucleic acid of interest.

Regarding claims 52 and 54, Jones teaches that the nucleic acid sequence of interest comprises genomic DNA (paragraph 100) or RNA (paragraphs 43-44).

Regarding claim 55, Jones teaches that the first and second adaptor sequences are distinct from each other (Figure 3, paragraph 87, and paragraph 100).

Further regarding claim 57, Jones teaches using a plurality of oligonucleotide to practice the capture and extension steps, and the plurality of oligonucleotides comprises a 3' portion that is complementary to a different nucleic acid sequence of interest in the nucleic acid fragments and a 5' portion comprising the same second adapter sequence (paragraphs 87, 99, and 100; see also Figure 3).

Regarding claim 58, Jones teaches that the second adaptor sequence may comprise a barcode sequence (paragraphs 58 and 61).

Regarding claim 59, Jones teaches that the nucleic acid fragments are double-stranded and that method comprises denaturing the nucleic acid fragments before the step of annealing the oligonucleotides (step (c) above), thereby generating single-stranded nucleic acid fragments comprising the first adaptor sequence and the nucleic acid sequence of interest (Figure 3 and paragraphs 87 and 100).

Jones does not teach massively parallel sequencing as required by independent claim 51. The reference teaches that nucleic acids amplified by the disclosed method may be analyzed by array hybridization or any other method known in the art to obtain sequence information about a region of interest (paragraph 79-80), but massively parallel sequencing is not disclosed. Therefore, the reference also does not meet the requirements of claims 61, 63, and 68-70, which further limit the sequencing step.

Tucker, though, describes methods of obtaining sequence information about a region of interest in a nucleic acid, and specifically describes a number of massively parallel sequencing methods (abstract, pages 142-146, and Figures 2-5). The reference states that “Massively parallel sequencing will allow simultaneous screening for mutations in hundreds of loci in genetically heterogeneous disorders, whole-genome screening for novel mutations, and sequence-based detection of novel pathogens that cause human disease. In addition, massively parallel sequencing will permit clinical application of our expanding knowledge of pharmacogenetics, cancer genetics, epigenetics, and complex traits” (page 142, column 2). The reference further teaches that massively parallel sequencing “has reduced the cost and increased the throughput of genomic sequencing by more than three orders of magnitude...and may also replace arrays and Sanger sequencing in clinical applications where they are currently being used” (abstract).

Further regarding claims 51, 61, and 68-70, Tucker describes a massively parallel sequencing method developed by Illumina (Fig. 2). The method comprises bridge amplification and the use of four labeled reversible terminators (Fig. 2). The Illumina method also comprises attaching an adaptor to each end of a nucleic acid fragment of interest and using the adaptors to hybridize the nucleic acid of interest to flow cell sequences (Fig. 2). Each of the two adaptors is also complementary to a sequencing primer (Fig. 2). Further regarding claim 63, Tucker notes that nanopore sequencing is another form massively parallel sequencing (page 143).

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to analyze the amplification products generated by the method of Jones using one of the massively parallel sequencing methods described in Tucker. The ordinary artisan would have been motivated to do so since Tucker taught that such methods offered very high-throughput sequencing (abstract and page 142) and as well as the ability to accurately detect

minor alleles (page 144, column 2). Additional motivation is provided in the abstract of Tucker, which teaches that massively parallel sequencing methods may replace array methods. The ordinary artisan would have had a reasonable expectation of success in view of the guidance provided by Tucker concerning the use of massively parallel sequencing methods and also since Jones taught that the amplification products could be analyzed using any desired method. Therefore, it would have been obvious to use any of the massively parallel sequencing methods disclosed in Tucker. The ordinary artisan would have had a reasonable expectation of success since many of the methods disclosed in Tucker have been commercialized and the reference provides guidance as to how the different sequencing methods are conducted. Lastly, when using the Illumina sequencing method, the ordinary artisan would have recognized that the 3' end of the amplification products should be complementary to a surface-immobilized sequence (i.e., as recited in claim 68) and, accordingly, would have been motivated to design the adaptors such that they are complementary to sequences on a support to be used in the Illumina sequencing method. Thus, the methods of claims 51, 52, 54-61, and 63-70 are *prima facie* obvious.

10. Claim 53 is rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jones et al. (US 2005/0142577 A1; IDS reference) in view of Tucker et al. (The American Journal of Human Genetics 2009; **85**: 142-154; IDS reference) and further in view of Gormley et al. (US 7,741,463 B2; IDS reference).

As discussed above, the teachings of Jones in view of Tucker render obvious the methods of claims 51, 52, 54-61, and 63-70.

Regarding claim 53, Jones does not teach that the nucleic acid fragment comprises cDNA. Tucker does not remedy this deficiency in Jones.

Gormley describes a method for preparing a library for sequencing that comprises fragmenting nucleic acids contained in a sample, purifying the fragments, end-repair of the purified fragments, ligation of a partial duplex adaptor, and PCR (see the working example at columns 23-26 and especially column 23, lines 25-67, column 25, line 41 – column 26, line 20, and column 26, lines 25-62; see also Figs. 2a-2b). Gormley further teaches using sequencing the nucleic acids in the library using a massively parallel method comprising the steps disclosed in Figure 2 of Tucker (i.e., bridge amplification and the use of reversible terminators) (column 20, line 18 – column 22, line 20). Further regarding claim 53, Gormley teaches that libraries for sequencing may be prepared from cDNA fragments (col. 6, ll. 5-27).

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to practice the method suggested by Jones in view of Tucker using cDNA fragments of interest prepared from RNA by reverse transcription. Gormley provides motivation to do so as well as a reasonable expectation of success since the teachings cited above indicate that cDNA libraries are a desirable template for massively parallel sequencing. Since it was routine and conventional to use reverse transcription to prepare cDNA, the teachings of Jones in view of Tucker and further in view of Gormley render obvious the method of claim 53.

11. Claim 62 is rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jones et al. (US 2005/0142577 A1; IDS reference) in view of Tucker et al. (The American Journal of Human Genetics 2009; **85**: 142-154; IDS reference) and further in view of Rothberg et al. (Nature 2011; **475**: 348-352 + Supplemental Information).

As discussed above, the teachings of Jones in view of Tucker render obvious the methods of claims 51, 52, 54-61, and 63-70.

Jones does not teach semiconductor sequences as recited in claim 62, and Tucker does not remedy this deficiency. Rothberg, however, describes massively parallel sequencing using a semiconductor device (Figure 1 and pages 348-350) and discloses that this sequencing method is highly robust, scalable, and advantageously does not require the use of optics or labeled nucleotides (abstract and page 348).

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to select semiconductor sequencing as described by Rothberg as the massively parallel sequencing method when practicing the method of Jones in view of Tucker. Rothberg provides motivation to do so by stating that the disclosed semiconductor sequencing method is robust, highly scalable, and requires neither optical detection nor modified nucleotides (abstract and page 348). The ordinary artisan would have had a reasonable expectation of success in view of Rothberg's guidance concerning practice of the method (pages 349-350 and the Supplemental Methods). Thus, the method of claim 62 is *prima facie* obvious.

Double Patenting

12. The non-statutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper time-wise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A non-statutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29

USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on non-statutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based e-Terminal Disclaimer may be filled out completely online using web-screens. An e-Terminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about e-Terminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

13. Claims 51-62 and 64-69 are rejected on the ground of non-statutory double patenting as being unpatentable over claims 1-37 of U.S. Patent No. 9,650,628 B2 (IDS reference).

Although the claims at issue are not identical, they are not patentably distinct from each other because the claims of the '628 patent teach or suggest all of the elements of the instant claims.

The instant claim 51 is drawn to a method for sequencing an enriched nucleic acid of interest. The method comprises: (a) obtaining a nucleic acid fragment comprising a first adaptor sequence; (b) annealing one or more oligonucleotides to the nucleic acid sequence of interest, wherein said oligonucleotides have a 3' portion at least ten nucleotides in length that binds to the nucleic acid sequence of interest and a 5' tail portion that comprises a second adaptor and is not complementary to the nucleic acid sequence of interest; (c) extending the oligonucleotides of step (b) to form extension products having a 5' second adaptor sequence, a sequence complementary to the nucleic acid sequence of interest, and a sequence complementary to the first adaptor; (d) amplifying the extension products of step (c) using a first primer that anneals to a complement of the first adaptor sequence and a second primer that anneals to a complement of the second adaptor sequence to enrich for the sequence of interest; and (e) sequencing the enriched nucleic acid of interest on a massively parallel sequencing platform.

Claims 1 and 23 of the '628 patent are also drawn to a method for sequencing an enriched nucleic acid of interest. The method recited in these claims includes all of the elements of the instant claim 51 as well as additional elements. For example, claim 1 of the '628 patent requires a post-ligation purification step that is not required by the instant claim 51, and claim 23 of the '628 patent requires a pre-sequencing purification step that is not required by the instant claim 51. Therefore, claims 1 and 23 of the '628 patent recite a species of the method more generically claimed in the instant claim 51. As discussed in MPEP 804 II.B.1, a species anticipates a more

generic claim, and the instant claim 51 is not patentably distinct from the claims of the '628 patent.

The limitations of the instant claims 52-54 are recited in claim 2 and 24 of the '628 patent.

The limitations of the instant claims 55-58 are recited in claims 1, 3, 4, and 7 of the '628 patent.

The limitations of the instant claim 59 are recited in claim 23 of the '628 patent.

The limitations of the instant claim 60 are recited in claims 5 and 37 of the '628 patent.

The limitations of the instant claims 61 and 62 are recited in claims 18, 19, 31, and 32 of the '628 patent.

The limitations of the instant claims 64-67 are recited in claim 1 of the '628 patent.

The limitations of the instant claims 68 and 69 are recited in claims 1 and 23 of the '628 patent.

Thus, the instant claims 51-62 and 64-69 are not patentably distinct from claims 1-37 of the '628 patent.

14. Claims 63 and 70 are rejected on the ground of non-statutory double patenting as being unpatentable over claims 1-37 of U.S. Patent No. 9,650,628 B2 (IDS reference) in view of Tucker et al. (The American Journal of Human Genetics 2009; **85**: 142-154; IDS reference).

As discussed above, the instant claims 51-62 and 64-69 are not patentably distinct from the claims of the '628 patent.

The claims of the '628 patent do not teach that nanopore sequencing is the massively parallel sequencing method as required by the instant claim 63.

The claims of the '628 patent also do not teach that the massively parallel sequencing method is conducted in a flow cell as required by the instant claim 70.

Tucker, though, describes a massively parallel sequencing method developed by Illumina (Fig. 2). The method comprises bridge amplification and the use of four labeled reversible terminators (Fig. 2). The Illumina method also comprises attaching an adaptor to each end of a nucleic acid fragment of interest and using the adaptors to hybridize the nucleic acid of interest to flow cell sequences (Fig. 2). Each of the two adaptors is also complementary to a sequencing primer (Fig. 2). Tucker also teaches that nanopore sequencing is another form massively parallel sequencing (page 143).

The ordinary artisan practicing the sequencing method recited in the claims of the '628 patent would have recognized from the teachings of Tucker that a flow cell was suitable for use as the surface upon which the sequencing reaction was conducted and also that nanopore sequencing was a suitable massively parallel sequencing method. Since it is *prima facie* obvious to select a known material or method based on its suitability for the intended purpose in the absence of unexpected results (MPEP 2144.07) and no such evidence has been presented, the instant claims 63 and 70 are not patentably distinct from the claims of the '628 patent in view of Tucker.

15. Claims 51-62 and 64-69 are rejected on the ground of non-statutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 10,036,012 B2 (IDS reference).

Although the claims at issue are not identical, they are not patentably distinct from each other because the claims of the '012 patent teach or suggest all of the elements of the instant claims.

The instant claim 51 is drawn to a method for sequencing an enriched nucleic acid of interest. The method comprises: (a) obtaining a nucleic acid fragment comprising a first adaptor sequence; (b) annealing one or more oligonucleotides to the nucleic acid sequence of interest, wherein said oligonucleotides have a 3' portion at least ten nucleotides in length that binds to the nucleic acid sequence of interest and a 5' tail portion that comprises a second adaptor and is not complementary to the nucleic acid sequence of interest; (c) extending the oligonucleotides of step (b) to form extension products having a 5' second adaptor sequence, a sequence complementary to the nucleic acid sequence of interest, and a sequence complementary to the first adaptor; (d) amplifying the extension products of step (c) using a first primer that anneals to a complement of the first adaptor sequence and a second primer that anneals to a complement of the second adaptor sequence to enrich for the sequence of interest; and (e) sequencing the enriched nucleic acid of interest on a massively parallel sequencing platform.

Claim 1 of the '012 patent is also drawn to a method for sequencing an enriched nucleic acid of interest. The method recited in these claims includes all of the elements of the instant claim 51 as well as additional elements. For example, the partial duplex adapter required by claim 1 of the '012 patent is not required by the instant claim 51. Therefore, claim 1 of the '012 patent recites a species of the method more generically claimed in the instant claim 51. As discussed in MPEP 804 II.B.1, a species anticipates a more generic claim, and the instant claim 51 is not patentably distinct from the claims of the '012 patent.

The limitations of the instant claims 52-54 are recited in claim 2-4 of the '012 patent.

The limitations of the instant claims 55-58 are recited in claims 1 and 5-7 of the '012 patent.

The limitations of the instant claim 59 are recited in claims 11 and 12 of the '012 patent.

The limitations of the instant claim 60 are recited in claim 13 of the '012 patent.

The limitations of the instant claims 61 and 62 are recited in claims 18 and 19 of the '012 patent.

The limitations of the instant claims 64-67 are recited in claims 1, 11, 14, 16, and 20 of the '012 patent.

The limitations of the instant claims 68 and 69 are recited in claim 1 of the '012 patent.

Thus, the instant claims 51-62 and 64-69 are not patentably distinct from claims 1-22 of the '012 patent.

16. Claims 63 and 70 are rejected on the ground of non-statutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 10,036,012 B2 (IDS reference) in view of Tucker et al. (The American Journal of Human Genetics 2009; **85**: 142-154; IDS reference).

As discussed above, the instant claims 51-62 and 64-69 are not patentably distinct from the claims of the '012 patent.

The claims of the '012 patent do not teach that nanopore sequencing is the massively parallel sequencing method as required by the instant claim 63.

The claims of the '012 patent also do not teach that the massively parallel sequencing method is conducted in a flow cell as required by the instant claim 70.

Tucker, though, describes a massively parallel sequencing method developed by Illumina (Fig. 2). The method comprises bridge amplification and the use of four labeled reversible terminators (Fig. 2). The Illumina method also comprises attaching an adaptor to each end of a nucleic acid fragment of interest and using the adaptors to hybridize the nucleic acid of interest to flow cell sequences (Fig. 2). Each of the two adaptors is also complementary to a sequencing

primer (Fig. 2). Tucker also teaches that nanopore sequencing is another form massively parallel sequencing (page 143).

The ordinary artisan practicing the sequencing method recited in the claims of the '012 patent would have recognized from the teachings of Tucker that a flow cell was suitable for use as the surface upon which the sequencing reaction was conducted and also that nanopore sequencing was a suitable massively parallel sequencing method. Since it is *prima facie* obvious to select a known material or method based on its suitability for the intended purpose in the absence of unexpected results (MPEP 2144.07) and no such evidence has been presented, the instant claims 63 and 70 are not patentably distinct from the claims of the '012 patent in view of Tucker.

Conclusion

17. No claims are currently allowable.

Rabinowitz et al. (US 2012/0270212 A1; IDS reference) is cited as reference of interest. This reference teaches amplification of a nucleic acid having a first adapter attached thereto with a tailed target-specific primer (see, e.g., Figures 1-8) but the disclosed method does not meet the requirements of steps (b) and (c) in the instant claim 51.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Angela Bertagna whose telephone number is (571)272-8291. The examiner can normally be reached on 8-5, M-F.

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Primary Examiner, Art Unit 1637

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	Filing Date		2018-06-25
	First Named Inventor		Douglas A. Amorese
	Art Unit		1637
	Examiner Name	A. M. Bertagna	
	Attorney Docket Number		NUGN-037/04US 34079/440

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/A.B./	3	20070231823	A1	2007-10-04	McKernan et al.		

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/A.B/	5	20090127589	A1	2009-05-21	Rothberg et al.	
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/A.B./	1	2009/112844	WO	A1	2009-09-17	Stichting Het NI Kanker I		
/A.B./	2	2011/009941	WO	A1	2011-01-27	Olink Genomics AB		
/A.B./	3	2011/156529	WO	A2	2011-12-15	Nugen Technologies, Inc		
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/A.B./	3	European search report and opinion mailed July 23, 2015, for EP Appl. 13740653 (9 pages)	
/A.B./	4	International Search Report and Written Opinion mailed April 16, 2013, for PCT/US2013/023278, filed Jan 25, 2013 (11 pages)	

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/A.B/	8	Tucker, 2009, Massively parallel sequencing: the next big thing in genetic medicine, Am J Human Genet 85:142-154

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	002	US-4469863	09-04-1984	TS'O; Paul O. P. et al.	
	003	US-4582877	04-15-1986	FAIRCHOK; William J. et al.	
	004	US-4876187	10-24-1989	DUCK; Peter et al.	
	005	US-4935357	06-19-1990	SZYBALSKI; Wacław	
	006	US-4942124	07-17-1990	CHURCH; George M [Us] et al.	
	007	US-4988617	01-29-1991	LANDEGREN; Ulf et al.	
	008	US-4996143	02-26-1991	HELLER; Michael J. et al.	
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	010	US-5034506	07-23-1991	SUMMERTON; James E. et al.	
	011	US-5035996	07-30-1991	HARTLEY; James L.	
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	013	US-5082830	01-21-1992	BRAKEL; Christine L. et al.	
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	015	US-5130238	07-14-1992	MALEK; Lawrence T. et al.	
	016	US-5169766	12-08-1992	SCHUSTER; David M. et al.	
	017	US-5171534	12-15-1992	SMITH; Lloyd M. et al.	
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	043	US-5641658	06-24-1997	ADAMS; Christopher P. et al.	
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Examiner Signature	/ANGELA M. BERTAGNA/	Date Considered	04/21/2020
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)			Application Number	16017340	
			Filing Date	06-25-2018	
			First Named Inventor	AMORESE; Doug	
			Art Unit	Not Yet Assigned	
			Examiner Name	Not Yet Assigned	
			Attorney Docket Number	25115-755.302	
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U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
/A.B/	045	US-5665549	09-09-1997	PINKEL; Daniel et al.	
	046	US-5667976	09-16-1997	VAN; Ness Jeffrey et al.	
	047	US-5667979	09-16-1997	BERRENS; Lubertus	
	048	US-5679512	10-21-1997	LANEY; Maureen et al.	
	049	US-5681726	10-28-1997	HUSE; William David et al.	
	050	US-5683879	11-04-1997	LANEY; Maureen et al.	
	051	US-5688648	11-18-1997	MATHIES; Richard A. et al.	
	052	US-5705628	01-06-1998	HAWKINS; Trevor	
	053	US-5708154	01-13-1998	SMITH; Steven S. et al.	
	054	US-5710028	01-20-1998	EYAL; Nurit et al.	
	055	US-5712126	01-27-1998	WEISSMAN; Sherman M. et al.	
	056	US-5716785	02-10-1998	VAN; Gelder Russell N. et al.	
	057	US-5726329	03-10-1998	JONES; David S. et al.	
	058	US-5750341	05-12-1998	MACEVICZ; Stephen C.	
	059	US-5759822	06-02-1998	CHENCHIK; Alex et al.	
	060	US-5763178	06-09-1998	CHIRIKJIAN; Jack G. et al.	
	061	US-5789206	08-04-1998	TAVTIGIAN; Sean V. et al.	
	062	US-5824517	10-20-1998	CLEUZIAT; Philippe et al.	
	063	US-5824518	10-20-1998	KACIAN; Daniel Louis et al.	
	064	US-5837832	11-17-1998	CHEE; Mark et al.	
	065	US-5876976	03-02-1999	RICHARDS; Rodney M. et al.	
/A.B/	066	US-5882867	03-16-1999	ULLMAN; Edwin F. et al.	

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		Number-Kind Code ² (if known)			
/A.B./	067	US-5888779	03-30-1999	KACIAN; Daniel Louis et al.	
	068	US-5888819	03-30-1999	GOELET; Philip et al.	
	069	US-5945313	08-31-1999	HARTLEY; James L. et al.	
	070	US-5952176	09-14-1999	MCCARTHY; Thomas Valentine et al.	
	071	US-5958681	09-28-1999	WETMUR; James G. et al.	
	072	US-5965409	10-12-1999	PARDEE; Arthur B. et al.	
	073	US-5969119	10-19-1999	MACEVICZ; Stephen C.	
	074	US-5972618	10-26-1999	BLOCH; Will	
	075	US-6004744	12-21-1999	GOELET; Philip et al.	
	076	US-6004745	12-21-1999	ARNOLD Jr.; Lyle J. et al.	
	077	US-6027889	02-22-2000	BARANY; Francis et al.	
	078	US-6027923	02-22-2000	WALLACE; Robert Bruce	
	079	US-6030774	02-29-2000	LANEY; Maureen et al.	
	080	US-6037152	03-14-2000	RICHARDS; Rodney M. et al.	
	081	US-6056661	05-02-2000	SCHMIDT; Michael Roland	
	082	US-6077674	06-20-2000	SCHLEIFER; Arthur et al.	
	083	US-6087103	07-11-2000	BURMER; Glenna C.	
	084	US-6090553	07-18-2000	MATSON; Robert S.	
	085	US-6090591	07-18-2000	BURG; James Lawrence et al.	
	086	US-6107023	08-22-2000	REYES; Gregory R. et al.	
/A.B./	087	US-6110709	08-29-2000	AUSUBEL; Frederick M. et al.	

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			First Named Inventor	AMORESE; Doug	
			Art Unit	Not Yet Assigned	
			Examiner Name	Not Yet Assigned	
			Attorney Docket Number	25115-755.302	
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/A.B/	088	US-6150112	11-21-2000	WEISSMAN; Sherman et al.	
	089	US-6159685	12-12-2000	PINKEL; Daniel et al.	
	090	US-6160105	12-12-2000	CUNNINGHAM; Mary Jane et al.	
	091	US-6169194	01-02-2001	THOMPSON; Michael et al.	
	092	US-6172208	01-09-2001	COOK; Alan Frederick	
	093	US-6174680	01-16-2001	MAKRIGIORGOS; Gerassimos M.	
	094	US-6190865	02-20-2001	JENDRISAK; Jerome J. et al.	
	095	US-6194211	02-27-2001	RICHARDS; Cynthia Ann et al.	
	096	US-6197557	03-06-2001	MAKAROV; Vladimir L. et al.	
	097	US-6210891	04-03-2001	NYREN; Paal et al.	
	098	US-6225109	05-01-2001	JUNCOSA; Robert D. et al.	
	099	US-6225451	05-01-2001	BALLINGER; Dennis G. et al.	
	100	US-6232104	05-15-2001	LISHANSKI; Alla et al.	
	101	US-6251639	06-26-2001	KURN; Nurith	
	102	US-6262490	07-17-2001	HSU; Kao-Yu et al.	
	103	US-6270961	08-07-2001	DRMANAC; Radoje	
	104	US-6280935	08-28-2001	MACEVICZ; Stephen C.	
	105	US-6287766	09-11-2001	NOLAN; John P. et al.	
	106	US-6287825	09-11-2001	WEISSMAN; Sherman et al.	
	107	US-6291170	09-18-2001	VAN; Gelder Russell N. et al.	
/A.B/	108	US-6306365	10-23-2001	RUOSLAHTI; Erkki et al.	

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/A.B/	109	US-6306597	10-23-2001	MACEVICZ; Stephen C.	
	110	US-6309843	10-30-2001	TIMMS; Kathy Lynn	
	111	US-6326142	12-04-2001	ROYER; Catherine A.	
	112	US-6335167	01-01-2002	PINKEL; Daniel et al.	
	113	US-6339147	01-15-2002	LUKHTANOV; Eugeny Alexander et al.	
	114	US-6440705	08-27-2002	STANTON Jr.; Vincent P. et al.	
	115	US-6449562	09-10-2002	CHANDLER; Van S. et al.	
	116	US-6582938	06-24-2003	SU; Xing et al.	
	117	US-6670461	12-30-2003	WENGEL; Jesper et al.	
	118	US-6686156	02-03-2004	KURN; Nurith	
	119	US-6692918	02-17-2004	KURN; Nurith	
	120	US-6770748	08-03-2004	IMANISHI; Takeshi et al.	
	121	US-6777180	08-17-2004	FISHER; Paul B. et al.	
	122	US-6794499	09-21-2004	WENGEL; Jesper et al.	
	123	US-6815164	11-09-2004	KURN; Nurith	
	124	US-6815167	11-09-2004	CROTHERS; Donald M. et al.	
	125	US-6825011	11-30-2004	ROMANTCHIKOV; Yuri	
	126	US-6833246	12-21-2004	BALASUBRAMANIAN; Shankar	
	127	US-6849404	02-01-2005	PARK; Han Oh [Kr] et al.	
	128	US-6858413	02-22-2005	KURN; Nurith	
/A.B/	129	US-6917726	07-12-2005	LEVENE; Michael J. et al.	

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/A.B/	130	US-6924104	08-02-2005	WEISMANN; Sherman et al.	
	131	US-6946251	09-20-2005	KURN; Nurith	
	132	US-7001724	02-21-2006	GREENFIELD; I. Lawrence	
	133	US-7033764	04-25-2006	KORLACH; Jonas et al.	
	134	US-7048481	05-23-2006	SUGATA; Shinsuke et al.	
	135	US-7052847	05-30-2006	KORLACH; Jonas et al.	
	136	US-7056676	06-06-2006	KORLACH; Jonas et al.	
	137	US-7060441	06-13-2006	BOURGET; Cecile et al.	
	138	US-7094536	08-22-2006	KURN; Nurith	
	139	US-7170050	01-30-2007	TURNER; Stephen et al.	
	140	US-7175982	02-13-2007	MCCARTHY; Thomas Valentine et al.	
	141	US-7176025	02-13-2007	KURN; Nurith et al.	
	142	US-7189512	03-13-2007	PORAT; Noga et al.	
	143	US-7211390	05-01-2007	ROTHBERG; Jonathan M. et al.	
	144	US-7244559	07-17-2007	ROTHBERG; Jonathan M. et al.	
	145	US-7244567	07-17-2007	CHEN; Yi-Ju et al.	
	146	US-7273730	09-25-2007	DU; Breuil Lastrucci Rusla M.	
	147	US-7276720	10-02-2007	ULMER; Kevin	
	148	US-7300755	11-27-2007	PETERSDORF; Effie W. et al.	
	149	US-7302146	11-27-2007	TURNER; Stephen et al.	
/A.B/	150	US-7313308	12-25-2007	TURNER; Stephen et al.	

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/A.B/	151	US-7315019	01-01-2008	TURNER; Stephen et al.	
	152	US-7323305	01-29-2008	LEAMON; John H. et al.	
	153	US-7335762	02-26-2008	ROTHBERG; Jonathan M. et al.	
	154	US-7361466	04-22-2008	KORLACH; Jonas et al.	
	155	US-7361468	04-22-2008	LIU; Guoying et al.	
	156	US-7402386	07-22-2008	KURN; Nurith et al.	
	157	US-7405281	07-29-2008	XU; Yue et al.	
	158	US-7414117	08-19-2008	SAITO; Isao et al.	
	159	US-7416844	08-26-2008	KORLACH; Jonas et al.	
	160	US-7462452	12-09-2008	WILLIAMS; John G. et al.	
	161	US-7462468	12-09-2008	WILLIAMS; John G. K. et al.	
	162	US-7476503	01-13-2009	TURNER; Stephen et al.	
	163	US-7476504	01-13-2009	TURNER; Stephen	
	164	US-7491498	02-17-2009	LAPIDUS; Stanley et al.	
	165	US-7501245	03-10-2009	QUAKE; Stephen et al.	
	166	US-7579153	08-25-2009	BRENNER; Sydney et al.	
	167	US-7741463	06-22-2010	GORMLEY; Niall A. et al.	
	168	US-7803550	09-28-2010	MAKAROV; Vladimir L. et al.	
	169	US-8017335	09-13-2011	SMITH; Geoffrey Paul	
	170	US-8053192	11-08-2011	BIGNELL; Helen et al.	
	171	US-8143001	03-27-2012	KURN; Nurith et al.	
/A.B/	172	US-8551709	10-08-2013	KURN; Nurith et al.	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)			Application Number	16017340	
			Filing Date	06-25-2018	
			First Named Inventor	AMORESE; Doug	
			Art Unit	Not Yet Assigned	
			Examiner Name	Not Yet Assigned	
			Attorney Docket Number	25115-755.302	
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U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
/A.B./	173	US-9284602	03-15-2016	ZHANG; David Yu et al.	
	174	US-20010000077	03-29-2001	ENGELHARDT; Dean L. et al.	
	175	US-20010031739	10-18-2001	DARE; Akintade Oyedele	
	176	US-20010034048	10-25-2001	KURN; Nurith	
	177	US-20010041334	11-15-2001	RASHTCHIAN; Ayoub et al.	
	178	US-20020028447	03-07-2002	LI; Wu Bo et al.	
	179	US-20020058270	05-16-2002	KURN; Nurith	
	180	US-20020115088	08-22-2002	KURN; Nurith	
	181	US-20020150919	10-17-2002	WEISMANN; Sherman et al.	
	182	US-20020155451	10-24-2002	MAKRIGIORGOS; Gerassimos M.	
	183	US-20020164628	11-07-2002	KURN; Nurith	
	184	US-20020164634	11-07-2002	PATIL; Nila et al.	
	185	US-20020197639	12-26-2002	SHIA; Michael A. et al.	
	186	US-20030017591	01-23-2003	KURN; Nurith	
	187	US-20030022207	01-30-2003	BALASUBRAMANIAN; Shankar et al.	
	188	US-20030082543	05-01-2003	SU; Xing et al.	
	189	US-20030087251	05-08-2003	KURN; Nurith	
	190	US-20030119150	06-26-2003	ANKENBAUER; Waltraud et al.	
	191	US-20030143555	07-31-2003	BOURGET; Cecile et al.	
	192	US-20030175780	09-18-2003	JONES; Douglas H.	
/A.B./	193	US-20030180779	09-25-2003	LOFTON-DAY CATHY E. et al.	

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/A.B/	194	US-20030186234	10-02-2003	KURN; Nurith	
	195	US-20030207279	11-06-2003	CROTHERS; Donald M. et al.	
	196	US-20030215926	11-20-2003	KURN; Nurith et al.	
	197	US-20030224439	12-04-2003	LAFFERTY; Mike et al.	
	198	US-20030232348	12-18-2003	JONES; Keith W. et al.	
	199	US-20040002371	01-01-2004	PAQUIN; Claude et al.	
	200	US-20040005614	01-08-2004	KURN; Nurith et al.	
	201	US-20040023271	02-05-2004	KURN; Nurith et al.	
	202	US-20040115815	06-17-2004	LI; Henry et al.	
	203	US-20040137456	07-15-2004	YOKOTA; Hiroki et al.	
	204	US-20040161742	08-19-2004	DEAN; Frank B. et al.	
	205	US-20040203019	10-14-2004	KURN; Nurith	
	206	US-20040203025	10-14-2004	KURN; Nurith	
	207	US-20040248153	12-09-2004	DEAR; Paul H. et al.	
	208	US-20050003441	01-06-2005	KURN; Nurith	
	209	US-20050014192	01-20-2005	KURN; Nurith	
	210	US-20050019793	01-27-2005	KURN; Nurith et al.	
	211	US-20050059048	03-17-2005	GUNDERSON; Kevin et al.	
	212	US-20050064456	03-24-2005	KURN; Nurith	
	213	US-20050123956	06-09-2005	BLUME; John E. et al.	
	214	US-20050136417	06-23-2005	COLE; Kyle B. et al.	
/A.B/	215	US-20050142577	06-30-2005	JONES; Keith W. et al.	

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/A.B/	216	US-20050191656	09-01-2005	DRMANAC; Radoje et al.	
	217	US-20050191682	09-01-2005	BARONE; Anthony D. et al.	
	218	US-20050208538	09-22-2005	KURN; Nurith et al.	
	219	US-20060008824	01-12-2006	RONAGHI; Mostafa et al.	
	220	US-20060014182	01-19-2006	KURN; Nurith	
	221	US-20060024678	02-02-2006	BUZBY; Philip R.	
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	223	US-20060035274	02-16-2006	DONG; Shoulia	
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	225	US-20060051789	03-09-2006	KAZAKOV; Sergei A. et al.	
	226	US-20060216724	09-28-2006	CHRISTIANS; Frederick C. et al.	
	227	US-20060263789	11-23-2006	KINCAID; Robert	
	228	US-20060281082	12-14-2006	ZHU; Jiahui	
	229	US-20060286566	12-21-2006	LAPIDUS; Stanley N. et al.	
	230	US-20060292597	12-28-2006	SHAPERO; Michael H. et al.	
	231	US-20070031857	02-08-2007	MAKAROV; Vladimir L. et al.	
	232	US-20070134128	06-14-2007	KORLACH; Jonas	
	233	US-20070224613	09-27-2007	STRATHMANN; Michael P.	
	234	US-20070231823	10-04-2007	MCKERNAN; Kevin J. et al.	
	235	US-20070238122	10-11-2007	ALLBRITTON; Nancy et al.	
	236	US-20080038727	02-14-2008	SPIER; Eugene G.	
/A.B/	237	US-20080087826	04-17-2008	HARRIS; Timothy D. et al.	

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/A.B/	238	US-20080103058	05-01-2008	SIDDIQI; Suhaib	
	239	US-20080176311	07-24-2008	KURN; Nurith	
	240	US-20080182300	07-31-2008	KURN; Nurith	
	241	US-20080194413	08-14-2008	ALBERT; Thomas J.	
	242	US-20080194416	08-14-2008	CHEN; Fuqiang	
	243	US-20080206764	08-28-2008	WILLIAMS; John G.K. et al.	
	244	US-20080213770	09-04-2008	WILLIAMS; Peter et al.	
	245	US-20080242560	10-02-2008	GUNDERSON; Kevin L. et al.	
	246	US-20080286795	11-20-2008	KAWASHIMA; Eric H. et al.	
	247	US-20090011959	01-08-2009	COSTA; Gina L. et al.	
	248	US-20090024331	01-22-2009	TOMANEY; Austin B. et al.	
	249	US-20090026082	01-29-2009	ROTHBERG; Jonathan M. et al.	
	250	US-20090029385	01-29-2009	CHRISTIANS; Fred et al.	
	251	US-20090061425	03-05-2009	LO; Yuk Ming Dennis et al.	
	252	US-20090061439	03-05-2009	BUZBY; Philip Richard	
	253	US-20090068645	03-12-2009	SIBSON; Ross	
	254	US-20090068655	03-12-2009	WILLIAMS; John G.K. et al.	
	255	US-20090068709	03-12-2009	KURN; Nurith et al.	
	256	US-20090117573	05-07-2009	FU; Glenn K. et al.	
	257	US-20090117621	05-07-2009	BOUTELL; Jonathan Mark et al.	
	258	US-20090124514	05-14-2009	FU; Glenn et al.	
/A.B/	259	US-20090127589	05-21-2009	ROTHBERG; Jonathan M. et al.	

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/A.B./	260	US-20090130721	05-21-2009	KURN; Nurith et al.	
	261	US-20090203085	08-13-2009	KURN; Nurith et al.	
	262	US-20090203531	08-13-2009	KURN; Nurith	
	263	US-20090233804	09-17-2009	KURN; Nurith et al.	
	264	US-20090239232	09-24-2009	KURN; Nurith	
	265	US-20090275486	11-05-2009	KURN; Nurith et al.	
	266	US-20090280538	11-12-2009	PATEL; Pranav et al.	
	267	US-20090298075	12-03-2009	TRAVERS; Kevin [Us] et al.	
	268	US-20100015666	01-21-2010	BRENNER; Sydney et al.	
	269	US-20100021973	01-28-2010	MAKAROV; Vladimir L. et al.	
	270	US-20100022403	01-28-2010	KURN; Nurith et al.	
	271	US-20100029511	02-04-2010	RAYMOND; Christopher K. et al.	
	272	US-20100105052	04-29-2010	DRMANAC; Radoje et al.	
	273	US-20100113296	05-06-2010	MYERSON; Joel	
	274	US-20100129879	05-27-2010	ACH; Robert A. et al.	
	275	US-20100137143	06-03-2010	ROTHBERG; Jonathan M. et al.	
	276	US-20100167954	07-01-2010	EARNSHAW; David James et al.	
	277	US-20100173394	07-08-2010	COLSTON Jr.; Billy Wayne et al.	
	278	US-20100273219	10-28-2010	MAY; Andrew et al.	
	279	US-20100323348	12-23-2010	HAMADY; Micah L [Us] et al.	
	280	US-20110015096	01-20-2011	CHIU; Kuo Ping	
/A.B./	281	US-20110039732	02-17-2011	RAYMOND; Christopher et al.	

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/A.B/	282	US-20110104785	05-05-2011	VAIDYANATHAN; Ramesh et al.	
	283	US-20110105364	05-05-2011	KURN; Nurith	
	284	US-20110129827	06-02-2011	CAUSEY; Marie et al.	
	285	US-20110189679	08-04-2011	KURN; Nurith et al.	
	286	US-20110224105	09-15-2011	KURN; Nurith et al.	
	287	US-20110288780	11-24-2011	RABINOWITZ; Matthew et al.	
	288	US-20110319290	12-29-2011	RAYMOND; Christopher et al.	
	289	US-20120045797	02-23-2012	KURN; Nurith et al.	
	290	US-20120071331	03-22-2012	CASBON; James et al.	
	291	US-20120074925	03-29-2012	OLIVER; John S.	
	292	US-20120102054	04-26-2012	POPESCU; Liviu et al.	
	293	US-20120122701	05-17-2012	RYAN; Allison et al.	
	294	US-20120157322	06-21-2012	MYLLYKANGAS; Samuel et al.	
	295	US-20120220483	08-30-2012	KURN; Nurith et al.	
	296	US-20120237943	09-20-2012	SOLDATOV; Aleksey et al.	
	297	US-20120245041	09-27-2012	BRENNER; Sydney et al.	
	298	US-20120270212	10-25-2012	RABINOWITZ; Matthew [Us] et al.	
	299	US-20120283145	11-08-2012	WANG; Yan	
	300	US-20120309002	12-06-2012	LINK; Darren	
	301	US-20130059738	03-07-2013	LEAMON; John et al.	
/A.B/	302	US-20140065692	03-06-2014	KURN; Nurith et al.	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Application Number	16017340
				Filing Date	06-25-2018
				First Named Inventor	AMORESE; Doug
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	15	of	61	Attorney Docket Number	25115-755.302

U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
/A.B./	303	US-20140274729	09-18-2014	KURN; Nurith et al.	
	304	US-20140274731	09-18-2014	RAYMOND; Christopher K [Us] et al.	
	305	US-20140274738	09-18-2014	AMORESE; Doug et al.	
	306	US-20140378345	12-25-2014	HINDSON; Benjamin et al.	
	307	US-20150011396	01-08-2015	SCHROEDER; Benjamin G. et al.	
	308	US-20150017635	01-15-2015	MYLLYKANGAS; Samuel et al.	
	309	US-20150284769	10-08-2015	SCHROEDER; Benjamin G. et al.	
	310	US-20150299767	10-22-2015	ARMOUR; Christopher et al.	
	311	US-20160040215	02-11-2016	HENN; Matthew R. et al.	
	312	US-20160130576	05-12-2016	ARMOUR; Chris	
	313	US-20160251712	09-01-2016	AMORESE; Doug et al.	
	314	US-20160265042	09-15-2016	SCHROEDER; Benjamin G. et al.	
/A.B./	315	US-20160362680-A1	12-15-2016	CHRISTOPHER; Armour et al.	

FOREIGN PATENT DOCUMENTS						
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		Country Code ³ Number ⁴ Kind Code ⁵ (if known)				
/A.B./	001	CA-2444926-A1	11-14-2002	CENTRE NAT RECH SCIENT [FR], et al.		☒

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		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B./	002	EP-0329822-B1	06-08-1994	CANGENE CORP [CA]		☐
/A.B./	003	EP-0365627-B1	12-22-1993	UNIV IOWA RES FOUND [US]		☐
/A.B./	004	EP-0667393-A2	08-16-1995	ENZO DIAGNOSTICS INC [US]		☐
/A.B./	005	EP-0667393-A3	11-15-1995	ENZO DIAGNOSTICS INC [US]		☐
/A.B./	006	EP-0843735-B1	07-17-2002	FORFAS TRADING AS BIORES IRELA [IE], et al.		☐
/A.B./	007	EP-1071811-B1	03-06-2002	ENTPR IE TRD AS BIORESEARCH IE [IE], et al.		☐
/A.B./	008	EP-2322612-A1	05-18-2011	HITACHI HIGH TECH CORP [JP], et al.		☐
/A.B./	009	WO-0008208-A2	02-17-2000	MEDICAL RES COUNCIL [GB], et al.		☐
/A.B./	010	WO-0008208-A3	05-11-2000	MEDICAL RES COUNCIL [GB], et al.		☐
/A.B./	011	WO-0009756-A1	02-24-2000	PERKIN ELMER CORP [US]		☐

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		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B/	012	WO-0018957-A1	04-06-2000	APPLIED RESEARCH SYSTEMS [NL], et al.		☐
/A.B/	013	WO-0039345-A1	07-06-2000	DANA FARBER CANCER INST INC [US], et al.		☐
/A.B/	014	WO-0052191-A1	09-08-2000	LIFE TECHNOLOGIES INC [US]		☐
/A.B/	015	WO-0055364-A2	09-21-2000	AMERSHAM PHARM BIOTECH UK LTD [GB], et al.		☐
/A.B/	016	WO-0055364-A3	10-11-2001	AMERSHAM PHARM BIOTECH UK LTD [GB], et al.		☐
/A.B/	017	WO-0120035-A2	03-22-2001	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	018	WO-0120035-A3	12-06-2001	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	019	WO-0123613-A1	04-05-2001	ISIS PHARMACEUTICALS INC [US], et al.		☐
/A.B/	020	WO-0146464-A1	06-28-2001	LION BIOSCIENCE AG [DE], et al.		☐
/A.B/	021	WO-0157248-A2	08-09-2001	SOLEXA LTD [GB]		☐

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		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B/	022	WO-0157248-A3	02-14-2002	SOLEXA LTD [GB]		☐
/A.B/	023	WO-0164952-A2	09-07-2001	MOLECULAR STAGING INC [US]		☐
/A.B/	024	WO-0164952-A3	12-27-2002	MOLECULAR STAGING INC [US]		☐
/A.B/	025	WO-0200938-A2	01-03-2002	NUGEN TGECHNOLOGIES INC [US], et al.		☐
/A.B/	026	WO-0200938-A3	08-28-2003	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	027	WO-02060318-A2	08-08-2002	UNIV OHIO STATE RES FOUND [US], et al.		☐
/A.B/	028	WO-02060318-A3	10-09-2003	UNIV OHIO STATE RES FOUND [US], et al.		☐
/A.B/	029	WO-02072772-A2	09-19-2002	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	030	WO-02072772-A3	09-12-2003	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	031	WO-02072773-A2	09-19-2002	NUGEN TECHNOLOGIES INC [US], et al.		☐

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/A.B/	032	WO-02072773-A3	12-18-2003	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	033	WO-02081753-A1	10-17-2002	ADVANCED RES & TECH INST [US], et al.		☐
/A.B/	034	WO-02090584-A2	11-14-2002	BIO MERIEUX [FR], et al.		☒
/A.B/	035	WO-02090584-A3	09-25-2003	BIO MERIEUX [FR], et al.		☒
/A.B/	036	WO-0228876-A2	04-11-2002	RIKEN [JP], et al.		☐
/A.B/	037	WO-0228876-A3	08-01-2002	RIKEN [JP], et al.		☐
/A.B/	038	WO-0229117-A2	04-11-2002	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	039	WO-0229117-A3	08-14-2003	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	040	WO-0236821-A2	05-10-2002	UNIV COLLEGE CORK NAT UNIV IE [IE], et al.		☐
/A.B/	041	WO-0236821-A3	03-27-2003	UNIV COLLEGE CORK NAT UNIV IE [IE], et al.		☐

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/A.B/	042	WO-0248402-A2	06-20-2002	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	043	WO-0248402-A3	04-08-2004	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	044	WO-03002736-A2	01-09-2003	ROCHE DIAGNOSTICS GMBH [DE], et al.		☐
/A.B/	045	WO-03012118-A1	02-13-2003	AFFYMETRIX INC [US], et al.		☐
/A.B/	046	WO-03027259-A2	04-03-2003	EPIGENX PHARMACEUTICALS INC [US], et al.		☐
/A.B/	047	WO-03027259-A3	12-11-2003	EPIGENX PHARMACEUTICALS INC [US], et al.		☐
/A.B/	048	WO-03078645-A2	09-25-2003	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	049	WO-03078645-A3	03-18-2004	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	050	WO-03083435-A2	10-09-2003	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	051	WO-03083435-A3	02-26-2004	NUGEN TECHNOLOGIES INC [US], et al.		☐

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/A.B./	052	WO-03106642-A2	12-24-2003	AFFYMETRIX INC [US], et al.		☐
/A.B./	053	WO-03106642-A3	11-04-2004	AFFYMETRIX INC [US], et al.		☐
/A.B./	054	WO-2004011665-A2	02-05-2004	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B./	055	WO-2004011665-A3	07-21-2005	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B./	056	WO-2005065321-A2	07-21-2005	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B./	057	WO-2005065321-A3	05-28-2009	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B./	058	WO-2006081222-A2	08-03-2006	COMPASS GENETICS LLC [US], et al.		☐
/A.B./	059	WO-2006081222-A3	02-22-2007	COMPASS GENETICS LLC [US], et al.		☐
/A.B./	060	WO-2007018601-A1	02-15-2007	RUBICON GENOMICS INC [US], et al.		☐
/A.B./	061	WO-2007030759-A2	03-15-2007	NUGEN TECHNOLOGIES INC [US], et al.		☐

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				Application Number	16017340
				Filing Date	06-25-2018
				First Named Inventor	AMORESE, Doug
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
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FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No. ¹	Foreign Patent Document	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T ⁶
		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B/	062	WO-2007030759-A3	06-21-2007	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	063	WO-2007052006-A1	05-10-2007	SOLEXA LTD [GB], et al.		☐
/A.B/	064	WO-2007057652-A1	05-24-2007	SOLEXA LTD [GB], et al.		☐
/A.B/	065	WO-2007136717-A1	11-29-2007	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	066	WO-2008005459-A2	01-10-2008	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	067	WO-2008005459-A3	02-21-2008	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	068	WO-2008015396-A2	02-07-2008	SOLEXA LTD [GB], et al.		☐
/A.B/	069	WO-2008033442-A2	03-20-2008	UNIV LELAND STANFORD JUNIOR [US], et al.		☐
/A.B/	070	WO-2008033442-A3	10-16-2008	UNIV LELAND STANFORD JUNIOR [US], et al.		☐
/A.B/	071	WO-2008115185-A2	09-25-2008	NIMBLEGEN SYSTEMS INC [US], et al.		☐

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/A.B/	072	WO-2008115185-A3	12-24-2008	NIMBLEGEN SYSTEMS INC [US], et al.		☐
/A.B/	073	WO-2009053039-A1	04-30-2009	ROCHE DIAGNOSTICS GMBH [DE], et al.		☐
/A.B/	074	WO-2009112844-A1	09-17-2009	STICHTING HET NL KANKER I [NL], et al.		☐
/A.B/	075	WO-2009120372-A2	10-01-2009	PACIFIC BIOSCIENCES CALIFORNIA [US], et al.		☐
/A.B/	076	WO-2009120372-A3	01-07-2010	PACIFIC BIOSCIENCES CALIFORNIA [US], et al.		☐
/A.B/	077	WO-2009120374-A2	10-01-2009	PACIFIC BIOSCIENCES CALIFORNIA [US], et al.		☐
/A.B/	078	WO-2009120374-A3	12-17-2009	PACIFIC BIOSCIENCES CALIFORNIA [US], et al.		☐
/A.B/	079	WO-2010003153-A2	01-07-2010	LIFE TECHNOLOGIES CORP [US], et al.		☐
/A.B/	080	WO-2010030683-A1	03-18-2010	ROSETTA INPHARMATICS LLC [US], et al.		☐

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		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B/	081	WO-2010039991-A2	04-08-2010	TEXAS A & M UNIV SYS [US], et al.		☐
/A.B/	082	WO-2010063711-A1	06-10-2010	ANGELETTI P IST RICHERCHE BIO [IT], et al.		☐
/A.B/	083	WO-2010064893-A1	06-10-2010	KEYGENE NV [NL], et al.		☐
/A.B/	084	WO-2010115154-A1	10-07-2010	FLUIDIGM CORP [US], et al.		☐
/A.B/	085	WO-2010129937-A2	11-11-2010	LIFE TECHNOLOGIES CORP [US], et al.		☐
/A.B/	086	WO-2011003630-A1	01-13-2011	MAX PLANCK GESELLSCHAFT [DE], et al.		☐
/A.B/	087	WO-2011009941-A1	01-27-2011	OLINK GENOMICS AB [SE], et al.		☐
/A.B/	088	WO-2011032053-A1	03-17-2011	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	089	WO-2011156529-A2	12-15-2011	NUGEN TECHNOLOGIES INC [US], et al.		☐
	000	WO-2012103154-A1	08-02-2012	NUGEN TECHNOLOGIES INC [US], et al.		☐

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		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B/	091	WO-2013059746-A1	04-25-2013	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	092	WO-2013177220-A1	11-28-2013	SCRIPPS RESEARCH INST [US], et al.		☐
	093	WO-2013181775-A2	12-27-2013	NUGEN TECHNOLOGIES INC [US], et al.		☐
/A.B/	094	WO-9207951-A1	05-14-1992	KOW YOKE WAH [US]		☐
/A.B/	095	WO-9318052-A1	09-16-1993	ISIS PHARMACEUTICALS INC [US]		☐
/A.B/	096	WO-9416090-A1	07-21-1994	MOLECULAR TOOL INC [US], et al.		☐
/A.B/	097	WO-9640998-A1	12-19-1996	PIONEER HI BRED INT [US]		☐
/A.B/	098	WO-9712061-A1	04-03-1997	EPICENTRE TECHNOLOGIES CORP [US]		☐
/A.B/	099	WO-9725416-A2	07-17-1997	NYFOTEK AS [NO], et al.		☐
/A.B/	100	WO-9725416-A3	10-02-1997	NYFOTEK AS [NO], et al.		☐

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		Country Code ² Number ⁴ Kind Code ⁵ (if known)				
/A.B./	101	WO-9806736-A1	02-19-1998	LIFE TECHNOLOGIES INC [US]		☐
/A.B./	102	WO-9838296-A1	09-03-1998	GESHER ISRAEL ADVANCED BIOTECS [IL], et al.		☐
/A.B./	103	WO-9844151-A1	10-08-1998	GLAXO GROUP LTD [GB], et al.		☐
/A.B./	104	WO-9910540-A1	03-04-1999	LOPEZ OSVALDO J [US], et al.		☐
/A.B./	105	WO-9911819-A1	03-11-1999	PERKIN ELMER CORP [US]		☐
/A.B./	106	WO-9942618-A1	08-26-1999	DADE BEHRING INC [US]		☐

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Sheet	27	of	61	Attorney Docket Number	25115-755.302

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T ²
/A.B./	001	AB Applied Biosystems. The solid 3 system enabling the next generation of science. Presentation. 2009.	☐
/A.B./	002	ADAMCZYK, et al. Synthesis of a Chemiluminescent Acridinium Hydroxylamine (AHA) for the Direct Detection of Abasic Sites in DNA. Org. Lett. 1999; 1(5):779-781.	☐
/A.B./	003	ADAMCZYK, et al. O-(Fluoresceinylmethyl) hydroxylamine (OFMHA): A Fluorescent Regent for Detection of Damaged Nucleic Acids. Bioorg. & Med. Chem. Lett. 1998; 8:3599-3602.	☐
/A.B./	004	ADESSI, et al., Solid phase DNA amplification: characterisation of primer attachment and amplification mechanisms. Nucleic Acids Research. 2000 Oct. 15. 28:(20): e87.	☐
/A.B./	005	Agilent Technologies. Agilent Technologies adds human exon kit to next-generation-sequencing target enrichment portfolio. GenomicsNews.com. Posted 2009 Sept. 23, 2009. Available at http://www.genomicsnews.com/index.aspx?ID=103607&sm=Agilent%20technologies%20adds%20human%20exo . Accessed 10/6/2009.	☐
/A.B./	006	AHMED. Sequencing of Low-Diversity Libraries. 02/28/2012. http://cofactorgenomics.com/sequencing-low-diversity-libraries/ .	☐
/A.B./	007	ALBERT, et al. Direct selection of human genomic loci by microarray hybridization. Nat Methods. 2007 Nov;4(11):903-5. Epub 2007 Oct 14.	☐
/A.B./	008	ALVARADO, et al. Multiplexed direct genomic selection (MDiGS): a pooled BAC capture approach for highly accurate CNV and SNP/INDEL detection. Nucleic Acids Res. 2014 Jun;42(10):e82. doi: 10.1093/nar/gku218. Epub 2014 Mar 20.	☐
	009	AMORESE, et al. Improved pathogen sequencing of host-pathogen RNA sequence. AGBT 2016. Advances in Genome Biology Technology Conference. February 10, 2016. Orlando, FL. Nugen Technologies. Poster Presentation. 2 pages.	☐

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/A.B./	010	ANISIMOVA, et al. Isolation, characterization and molecular cloning of duplex-specific nuclease from the hepatopancreas of the kamchatka crab. BMC Biochemistry. 2008 May 21. 9:14 doi10.1186/1471-2091-9-14.	☐
/A.B./	011	ANTSON, et al. PCR-generated padlock probes detect single nucleotide variation in genomic DNA. Nucleic Acids Res. 2000 Jun 15;28(12):E58.	☐
/A.B./	012	ANWAR, et al. A stem-loop-mediated reverse transcription real-time PCR for the selective detection and quantification of the replicative strand of an RNA virus. Anal Biochem. 2006 May 1;352(1):120-8. Epub 2006 Feb 17.	☐
/A.B./	013	ARCHER, et al. Selective and flexible depletion of problematic sequences from RNA-seq libraries at the cDNA stage. BMC Genomics. 2014 May 26;15:401. doi: 10.1186/1471-2164-15-401.	☐
/A.B./	014	Arraystar, Inc. Arraystar Directional RNA-seq Prep Kit (dUTP Based). Cat#: A1208. 04/08/2013.	☐
/A.B./	015	AUSUBEL, et al. eds. Current Protocols in Molecular Biology. Wiley. 1987.	☐
/A.B./	016	BAIRD, et al. Rapid SNP discovery and genetic mapping using sequenced RAD markers. PLoS One. 2008;3(10):e3376.	☐
/A.B./	017	BALLESTAR, et al. Methyl-CpG-binding proteins. Targeting specific gene repression. Eur J Biochem 2001; 268:1-6.	☐
/A.B./	018	BANGS LABORATORIES, Inc. TechNote 205 retrieved at: http://www.bangslab.com/technotes/205.pdf . Visited on July 16, 2003. (8 pages).	☐
/A.B./	019	BASHIARDES, et al. Direct genomic selection. Nat Methods. 2005 Jan;2(1):63-9.	☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Application Number	16017340
		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
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NON-PATENT LITERATURE DOCUMENTS			
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/A.B/	020	BEUCAGE, et al. The functionalization of oligonucleotides via phosphoramidite derivative. Tetrahedron. 1993;49(10):1925-63.	☐
/A.B/	021	BEIER, et al. HT sequencing in biomedicine - new approaches in preparing samples. LABORWELT. Jan. 9, 2008.	☐
/A.B/	022	BEN-ARTZI, et al. Double-stranded RNA-dependent RNase activity associated with human immunodeficiency virus type 1 reverse transcriptase. Proc Natl Acad Sci U S A. 1992 Feb 1;89(3):927-31.	☐
/A.B/	023	BENTLEY, D. R. Whole-genome re-sequencing. Curr Opin Genet Dev. 2006 Dec;16(6):545-52. Epub 2006 Oct 18.	☐
/A.B/	024	BHATTACHARJEE, et al. Complementing next generation sequencing technologies with Agilent's SureSelect DNA capture array. Agilent. 2009 July 13.	☐
/A.B/	025	BIBIKOVA, et al. Targeted chromosomal cleavage and mutagenesis in drosophila using zinc-finger nucleases genetics. Genetics. 2002 July. 161: 1169-1175.	☐
/A.B/	026	Bioo Scientific. Illumina RNA-Seq Library Prep. Available at http://www.biooscientific.com/ProductsServices/NextGenSequencing/Illumina-Compatible/RNA-Seq.aspx . Accessed 06/16/2014.	☐
/A.B/	027	Bioo Scientific. NEXTflex RNA-Seq Kit. Available at http://www.biooscientific.com/ProductsServices/NextGenSequencing/Illumina-Compatible/RNA-Seq/NEXTflex%E2%84%A2RNA-SeqKit.aspx . Accessed 06/16/2014.	☐
/A.B/	028	BLOW, N. Genomics: catch me if you can. Nature Methods. 2009 July. 6:7.539-544.	☐

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/A.B./	029	BORMANN, et al. Whole methylome analysis by ultra-deep sequencing using two-base encoding. PLoS One. 2010 Feb 22;5(2):e9320.	☐
/A.B./	030	BORODINA, et al. A strand-specific library preparation protocol for RNA sequencing. Methods Enzymol. 2011;500:79-98. doi: 10.1016/B978-0-12-385118-5.00005-0.	☐
/A.B./	031	BOTURYN, et al. A simple and Sensitive Method for in Vitro Quantitation of Abasic Sites in DNA. Chem. Res. Toxicol. 1999; 12:476-482.	☐
/A.B./	032	BOTURYN, et al. Synthesis of Fluorescent Probes for the Detection of Abasic Sites in DNA. Tetrahedron. 1997; 53(15):5485-5492.	☐
/A.B./	033	BOWER, et al. Targeted rapid amplification of cDNA ends (T-RACE)--an improved RACE reaction through degradation of non-target sequences. Nucleic Acids Res. 2010 Nov;38(21):e194. doi: 10.1093/nar/gkq816. Epub 2010 Sep 15.	☐
/A.B./	034	BRADFORD, et al. A comparison of massively parallel nucleotide sequencing with oligonucleotide microarrays for global transcription profiling. BMC Genomics. 2010 May 5;11:282. doi: 10.1186/1471-2164-11-282.	☐
/A.B./	035	BRIGGS, et al. Targeted retrieval and analysis of five Neandertal mtDNA genomes. Science. 2009 Jul 17;325(5938):318-21. doi: 10.1126/science.1174462.	☐
/A.B./	036	BRILL et al. Synthesis of oligodeoxynucleoside phosphorodithioates via thioamidites. J. Am. Chem. Soc. 111:2321-2322 (1989).	☐
/A.B./	037	BROUDE. Stem-loop oligonucleotides: a robust tool for molecular biology and biotechnology. Trends Biotechnol. 2002 Jun;20(6):249-56.	☐

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/A.B./	038	BROWN, T.A. Ed. Molecular Biology, LabFax. Bios Scientific Publishers. Academic Press. 1991; pp. 147-148.	☐
/A.B./	039	BUCHMAN, et al. Selective RNA amplification: a novel method using dUMP-containing primers and uracil DNA glycosylase. PCR Methods Appl. 1993 Aug;3(1):28-31.	☐
/A.B./	040	BURROWS, et al. Oxidative Nucleobase Modifications Leading to Strand Scission. Chem Rev. 1998 May 7;98(3):1109-1151.	☐
/A.B./	041	CALLOW, et al. Selective DNA amplification from complex genomes using universal double-sided adapters. Nucleic Acids Res. 2004 Jan 28;32(2):e21.	☐
/A.B./	042	CAREY, et al. Human Apurinic/Apyrimidinic Endonuclease in Processive. Biochem. 1999; 38:16553-16560.	☐
/A.B./	043	CARLSSON et al. Screening for genetic mutations. Nature 380(6571):207 (1996).	☐
/A.B./	044	CHAN, et al. The biophysics of DNA hybridization with immobilized oligonucleotide probes. Biophys J. 1995 Dec;69(6):2243-55.	☐
/A.B./	045	CHEN, et al. BisQC: an operational pipeline for multiplexed bisulfite sequencing. BMC Genomics. 2014 Apr 16;15:290. doi: 10.1186/1471-2164-15-290.	☐
/A.B./	046	CHEN, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005 Nov 27;33(20):e179.	☐
/A.B./	047	CHENCHIK, et al. Full-length cDNA cloning and determination of mRNA 5' and 3' ends by amplification of adaptor-ligated cDNA. Biotechniques. 1996 Sep;21(3):526-34.	☐

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/A.B/	048	Clontech Laboratories, Inc. In-Fusion SMARTer Directional cDNA Library Construction Kit User Manual. Cat. No. 634933. Copyright 2013.	☐
/A.B/	049	CNV detection by ion semiconductor sequencing. Life Technologies. 2014.	☐
/A.B/	050	COFACTOR genomics. Directional RNA Sequencing. Available at http://cofactorgenomics.com/directional-rna-sequencing . Accessed 06/04/2014.	☐
/A.B/	051	Combined search and examination report dated 4/24/2013 for GB1305340.	☐
/A.B/	052	CRAIG, et al. Identification of genetic variants using bar-coded multiplexed sequencing. Nat Methods. 2008 Oct;5(10):887-93. doi: 10.1038/nmeth.1251. Epub 2008 Sep 14.	☐
/A.B/	053	CROUCHER, et al. A simple method for directional transcriptome sequencing using Illumina technology. Nucleic Acids Res. 2009 Dec;37(22):e148.	☐
/A.B/	054	DAHL, et al. Multigene amplification and massively parallel sequencing for cancer mutation discovery. Proc Natl Acad Sci U S A. 2007 May 29;104(22):9387-92. Epub 2007 May 17.	☐
/A.B/	055	DAHL, et al. Multiplex amplification enabled by selective circularization of large sets of genomic DNA fragments. Nucleic Acids Res. 2005 Apr 28;33(8):e71.	☐
/A.B/	056	DEMPCY et al. Synthesis of a thymidyl pentamer of deoxyribonucleic guanidine and binding studies with DNA homopolynucleotides PNAS USA 92:6097-6101 (1995).	☐
/A.B/	057	DERISI et al. Use of cDNA microarray to analyse gene expression patterns in human cancer. Nature Genetics. 1996;14:457-460.	☐

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/A.B./	058	Diagnosing problems with phasing and pre-phasing on Illumina platforms. Loman Labs. 11/21/2013. http://nickloman.github.io/high-throughput%20sequencing/2013/11/21/diagnosing-problems-with-phasing-and-pre-phasing-on-illumina-platforms/ .	☐
/A.B./	059	DRESSMAN, et al. "Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations." PNAS, July 2003, Vol. 100. No. 15, 8817-8822.	☐
/A.B./	060	DRMANAC, et al. Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. Science. 2010 Jan 1;327(5961):78-81. doi: 10.1126/science.1181498. Epub 2009 Nov 5.	☐
/A.B./	061	EGHOLM et al. Peptide nucleic acids (PNA) oligonucleotide analogues with an achiral peptide backbone. J Am Chem Soc 114:1895-1897 (1992).	☐
/A.B./	062	EGHOLM et al. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature 365(6446):566-568 (1993).	☐
/A.B./	063	ERLANGER, et al. Antibodies Specific For Ribonucleosides And Ribonucleotides And Their Reaction With DNA. Proc Natl Acad Sci USA. 1964; 52:68-74.	☐
/A.B./	064	ESTELLER, Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet. 2007 Apr;8(4):286-98. Epub 2007 Mar 6.	☐
/A.B./	065	European office action dated 04/01/2011 for Application No. 03771533.1.	☐
/A.B./	066	European search report and opinion dated 05/22/2015 for EP Application No. 12842163.3.	☐
/A.B./	067	European search report and opinion dated 07/23/2015 for EP Application No. 13740653.	☐

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/A.B./	068	European search report and opinion dated 09/01/2016 for EP Application No. 14764629.3.	☐
/A.B./	069	European search report and opinion dated 11/28/2013 for EP Application No. 11793123.8.	☐
/A.B./	070	European search report and search opinion dated 04/03/2013 for Application No. 10808789.1.	☐
/A.B./	071	European search report dated 10/18/2007 for Application No. 3771533.1.	☐
/A.B./	072	European search report dated 2/12/10 for Application No. 7810169.8.	☐
/A.B./	073	European search report dated 3/29/10 for Application No. 4815722.6.	☐
/A.B./	074	FADROSH, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome. 2014 Feb 24;2(1):6. doi: 10.1186/2049-2618-2-6.	☐
/A.B./	075	FAHY, et al., Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR. Genome Res. 1991. 1:25-33.	☐
/A.B./	076	FAIRCLOTH, et al. Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. PLoS One. 2012;7(8):e42543. doi: 10.1371/journal.pone.0042543. Epub 2012 Aug 10.	☐
/A.B./	077	FEINBERG, et al. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature. 1983 Jan 6;301(5895):89-92.	☐
/A.B./	078	FODOR et al. Light-Directed, spatially addressable parallel chemical synthesis. Science. 1991;251:767-773.	☐

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/A.B./	079	FRANCA, et al. Optimizing a qPCR gene expression quantification assay for S. epidermidis biofilms: a comparison between commercial kits and a customized protocol. PLoS One. 2012;7(5):e37480. doi: 10.1371/journal.pone.0037480. Epub 2012 May 21.	☐
/A.B./	080	FRANK. BARCRAWL and BARTAB: software tools for the design and implementation of barcoded primers for highly multiplexed DNA sequencing. BMC Bioinformatics. 2009 Oct 29;10:362.	☐
/A.B./	081	FREDRIKSSON, et al. Multiplex amplification of all coding sequences within 10 cancer genes by Gene-Collector. Nucleic Acids Res. 2007;35(7):e47. Epub 2007 Feb 22.	☐
/A.B./	082	FREEMAN, et al. Fundamentals of DNA Hybridization Arrays for Gene Expression Analysis. BioTechniques. November 2000; 29:1042-1044, 1046, 1048-1055.	☐
/A.B./	083	FRESHNEY, R.I. ed. (1987). Animal Cell Culture. IRL Press: Oxford, pp. vii-xii (Table of Contents Only).	☐
/A.B./	084	FUJIWARA, et al. Direct probing: covalent attachment of probe DNA to double-stranded target DNA. Nucleic Acids Res. 1998 Dec 15;26(24):5728-33.	☐
/A.B./	085	FULLWOOD, et al. Next Generation DNA sequencin of paired-end tags (PET) for transcriptome and genome analyses. Genome Research. 19(4):521-532 (April 2009).	☐
/A.B./	086	GAIT, M.J., Ed. 1984 . Oligonucleotide Synthesis: A Practical Approach. IRL Press: Oxford, pp. vii-xii (Table of Contents).	☐
/A.B./	087	GAANOVA-RAEVA, et al. Primer Extension Enrichment Reaction (PEER): a new subtraction method for identification of genetic differences between biological specimens. Nucleic Acids Research. 2006; 34(11):e76.	☐

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/A.B/	088	GERRISH, et al. Tailed pooled suppression subtractive hybridization (PSSH) adaptors do not alter efficiency. Antonie Van Leeuwenhoek. 2010 Nov;98(4):573-9. doi: 10.1007/s10482-010-9465-x. Epub 2010 Jun 8.	☐
/A.B/	089	GERTZ, et al. Transposase mediated construction of RNA-seq libraries. Genome Res. 2012 Jan;22(1):134-41. doi: 10.1101/gr.127373.111. Epub 2011 Nov 29.	☐
/A.B/	090	GHOSH, S.S. Synthesis of 5'-Oligonucleotide Hydrazide Derivatives and Their Use in Preparation of Enzyme-Nucleic Acid Hybridization Probes. Anal. Biochem. 1989; 178:43-51.	☐
/A.B/	091	GNIRKE, et al. Solution Hybrid Selection with Ultra-long Oligonucleotides for Massively Parallel Targeted Sequencing. Nat. Biotechnol. (2009), 27(2):182-9.	☐
/A.B/	092	GU, et al. Partitioning the c. elegans genome by nucleosome modification, occupancy, and position. Online 2009 August 25. http://www.springerlink.com/content/r0gw044155823242/fulltext.pdf . Accessed October 6, 2009.	☐
/A.B/	093	GU, et al. Depletion of Abundant Sequences by Hybridization (DASH): using Cas9 to remove unwanted high-abundance species in sequencing libraries and molecular counting applications. Genome Biology. 2016; 17:41. DOI: 10.1186/s13059-016-0904-5.	☐
/A.B/	094	GU, et al. Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. Nat Protoc. 2011 Apr;6(4):468-81. doi: 10.1038/nprot.2010.190. Epub 2011 Mar 18.	☐
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/A.B/	096	HEIMGARTNER, et al. Polyacrylic Polyhydrazides as Reagents for Detection of Glycoproteins. Anal. Biochem. 1989; 181:182-189.	☐

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Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Complete if Known			
		Application Number	16017340		
		Filing Date	06-25-2018		
		First Named Inventor	AMORESE; Doug		
		Art Unit	Not Yet Assigned		
		Examiner Name	Not Yet Assigned		
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/A.B./	097	HODGES, et al. Genome-wide in situ exon capture for selective resequencing. Nat Genet. 2007 Dec;39(12):1522-7. Epub 2007 Nov 4.	☐
/A.B./	098	HODGES, et al. Hybrid selection of discrete genomic intervals on custom-designed microarrays for massively parallel sequencing. Nat. Protoc. 2009; 4(6): 960-974.	☐
/A.B./	099	HOLLIS, et al. Structural studies of human alkyladenine glycosylase and E. coli 3-methyladenine glycosylase. Mutat Res. 2000; 460(3-4):201-10.	☐
/A.B./	100	HORN, et al. Solid supported hydrolysis of apurinic sites in synthetic oligonucleotides for rapid and efficient purification on reverse-phase cartridges. Nucl. Acids Res. 1988; 16:11559-11571.	☐
/A.B./	101	HOTTIGER, et al. Strand displacement activity of the human immunodeficiency virus type 1 reverse transcriptase heterodimer and its individual subunits. J Biol Chem. 1994 Jan 14;269(2):986-91.	☐
/A.B./	102	HUBER, et al. Processing of the primer for plus strand DNA synthesis by human immunodeficiency virus 1 reverse transcriptase. J Biol Chem. 1990 Jun 25;265(18):10565-73.	☐
/A.B./	103	HURD, et al. Advantages of next-generation sequencing versus the microarray in epigenetic research. Brief Funct Genomic Proteomic. 2009 May;8(3):174-83. doi: 10.1093/bfgp/elp013. Epub 2009 Jun 17.	☐
/A.B./	104	IDE, et al. Synthesis and Damage Specificity of a Novel Probe for the Detection of Abasic Sites in DNA. Biochem. 1993; 32:8276-8283.	☐
/A.B./	105	illumina Inc. Directional mRNA-Seq Sample Preparation - Application to prepare directional (strand specific) sample from mRNA. October 2010.	☐

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/A.B./	106	International Preliminary Examination Report mailed on March 22, 2006 for PCT Patent Application No. PCT/US03/15825 filed May 19, 2003, 9pages.	☐
/A.B./	107	International search report and written opinion dated 01/27/2012 for PCT Application No. US2011/039683.	☐
/A.B./	108	International search report and written opinion dated 02/12/2013 for PCT/US2012/061218.	☐
/A.B./	109	International search report and written opinion dated 02/24/2011 for PCT Application No. US10/55137.	☐
/A.B./	110	International search report and written opinion dated 04/16/2013 for PCT Application No. US2013/023278.	☐
/A.B./	111	International search report and written opinion dated 05/10/2012 for PCT Application No. US2012/22448.	☐
/A.B./	112	International search report and written opinion dated 06/18/2015 for PCT/US2014/018112.	☐
/A.B./	113	International search report and written opinion dated 07/15/2014 for PCT Application No. US2014/028356.	☐
/A.B./	114	International search report and written opinion dated 07/29/2014 for PCT Application No. US2014/24581.	☐
/A.B./	115	International search report and written opinion dated 10/18/2013 for PCT Application No. US2013/032606.	☐
/A.B./	116	International search report and written opinion dated 12/3/2010 for PCT Application No. US10-45384.	☐

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/A.B/	117	International search report dated 1/2/2008 for PCT Application No. US2007/15409.	☐
/A.B/	118	International search report dated 6/14/2005 for PCT Application No. US 2003/015825.	☐
/A.B/	119	International search report dated 7/9/2008 for PCT Application No. US2004/043710.	☐
/A.B/	120	JENKINS et al. The Biosynthesis of Carbocyclic Nucleosides Chem Soc Re 24:169-176 (1995).	☐
/A.B/	121	JONES, et al. The epigenomics of cancer. Cell. 2007 Feb 23;128(4):683-92.	☐
/A.B/	122	KABOEY, et al. PCR hot start using primers with the structure of molecular beacons (hairpin-like structure). Nucleic Acids Res. 2000 Nov 1;28(21):E94.	☐
/A.B/	123	KARATA, et al. Construction of a circular single-stranded DNA template containing a defined lesion. DNA Repair (Amst). 2009 Jul 4;8(7):852-6.	☐
/A.B/	124	KAROW. New Capture Method Enables MPI Team to Sequence Five Neandertal Mitochondrial Genomes. GenomeWeb. July 21, 2009. https://www.genomeweb.com/sequencing/new-capture-method-enables-mpi-team-sequence-five-neandertal-mitochondrial-genom .	☐
/A.B/	125	KAWARADA, et al. Antibodies Specific for Methylated DNA Elicited in Rabbits Recognize only a Single Strand Region of DNA Containing 7-Methylguanine. Tohoku. J Exp Med. 1986; 149:151-161.	☐
/A.B/	126	KHRAPKO, et al. A method for DNA sequencing by hybridization with oligonucleotide matrix. DNA Sequence- J. DNA Sequencing and Mapping. 1991; 1:375-388.	☐
/A.B/	127	KIEDROWSKI, et al. Parabolic growth of a self-replicating hexadeoxynucleotide bearing a 3'-5'-phosphoamidate linkage. Angew. Chem. Intl. Ed. English 1991;30:423-426.	☐

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/A.B./	128	KIM, et al. Evidence for thiol-dependent production of oxygen radicals by 4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione (oltipraz) and 3H-1,2-dithiole-3-thione: possible relevance to the anticarcinogenic properties of 1,2-dithiole-3-thiones. Chem Res Toxicol. 1997 Mar;10(3):296-301.	☐
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/A.B./	130	KOW, et al. Detection of Abasic Sites and Oxidative DNA Base Damage Using an ELISA-like Assay. Methods. 2000; 22:164-169.	☐
/A.B./	131	KOZICH et al. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 79(17):5112-5120 (2013).	☐
/A.B./	132	KRISHNAKUMAR, et al. A comprehensive assay for targeted multiplex amplification of human DNA sequences. Proc Natl Acad Sci U S A. 2008 Jul 8;105(27):9296-301. doi: 10.1073/pnas.0803240105. Epub 2008 Jul 2.	☐
/A.B./	133	KRUEGER, et al. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics. 2011 Jun 1;27(11):1571-2. doi: 10.1093/bioinformatics/btr167. Epub 2011 Apr 14.	☐
/A.B./	134	KRUEGER, et al. Large scale loss of data in low-diversity illumina sequencing libraries can be recovered by deferred cluster calling. PLoS One. 2011 Jan 28;6(1):e16607. doi: 10.1371/journal.pone.0016607.	☐
/A.B./	135	KRUEGER. Loss of data in low-diversity libraries can be recovered by deferred cluster calling. Poster 01/29/2011. http://seqanswers.com/forums/showthread.php?t=9150 .	☐

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/A.B./	136	KUBO, et al. A Novel Sensitive, and Specific Assay for Abasic Sites, the Most Commonly Produced DNA Lesion. Biochem. 1992; 31:3703-3708.	☐
/A.B./	137	KUMAR, et al. A High-Throughput Method for Illumina RNA-Seq Library Preparation. Front Plant Sci. 2012 Aug 28;3:202. doi: 10.3389/fpls.2012.00202. eCollection 2012.	☐
/A.B./	138	KURN. Method for generation of double stranded cDNA from RNA targets useful for global amplification, sequencing or other quantification of short RNA in a sample. March 21, 2010. 1-5.	☐
/A.B./	139	LAIRD. Principles and challenges of genomewide DNA methylation analysis. Nat Rev Genet. 2010 Mar;11(3):191-203. doi: 10.1038/nrg2732.	☐
/A.B./	140	LAO, et al. Real time PCR profiling of 330 human micro-RNAs. Biotechnol J. 2007 Jan;2(1):33-5.	☐
/A.B./	141	LC Sciences. Targeted sequencing - sample enrichment service. 2009. Available at www.lcsciences.com/products/genomics/targeted_sequencing/targeted_sequencing.html . Accessed October 6, 2009.	☐
/A.B./	142	LC Sciences. Technology - Massively parallel oligonucleotide and peptide synthesis on a microchip based on the uParaflo microfluidic technology. Available at www.lcsciences.com/support/technology/technology.html . Accessed October 6, 2009.	☐
/A.B./	143	LC Sciences. Oligonucleotide mixture. OligoMix. 2009. Available at www.lcsciences.com/products/genomics/oligomix/oligomix_detail.html . Accessed October 6, 2009.	☐
/A.B./	144	LEAMON, et al., a Massively parallel Pico TiterPlate based platform for discrete picoliter-scale polymerase chain reactions [abstract]. Electrophoresis. 2003 Nov. 24(21) 3769-77.	☐

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/A.B./	145	LEFRANCOIS, et al. Efficient yeast ChIP-Seq using multiplex short-read DNA sequencing. BMC Genomics. 2009 Jan 21;10:37.	☐
/A.B./	146	LENNON, et al. A scalable, fully automated process for construction of sequence-ready barcoded libraries for 454. Genome Biol. 2010;11(2):R15.	☐
/A.B./	147	LEONARD. What is a reliable method for multiplexing more than 384 samples on a MiSeq run? Posted 08/19/2013. http://www.researchgate.net/post/What_is_a_reliable_method_for_multiplexing_more_than_384_samples_on_a_MiSeq_run2 .	☐
/A.B./	148	LETSINGER et al. Cationic Oligonucleotides J Am Chem Soc 110:4470-4471 (1988).	☐
/A.B./	149	LETSINGER et al. Effects of pendant groups at phosphorus on binding properties of d-ApA analogues. Nucl Acids Res 14(8):3487-3499 (1986).	☐
/A.B./	150	LETSINGER et al. Phosphoramidate Analogs of Oligonucleotides J Org Chem 35(11):3800-3803 (1970).	☐
/A.B./	151	LEVIN, et al. Comprehensive comparative analysis of strand-specific RNA sequencing methods. Nat Methods. 2010 Sep;7(9):709-15. doi: 10.1038/nmeth.1491. Epub 2010 Aug 15.	☐
/A.B./	152	LHOMME, et al. Abasic DNA Structure reactivity and recognition. Biopolymers. 1999; 52(2): 65-83.	☐
	153	LI, et al. Targeted depletion of host reads in host-pathogen mixed RNA-seq libraries. Advances in Genome Biology Technology Conference. February 11, 2016. Orlando, FL. Nugen Technologies. Poster. 1 page.	☐

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/A.B./	154	LINDAHL, T. An N-Glycosidase from Escherichia coli That Releases Free Uracil from DNA Containing Deaminated Cytosine Residues. Proc Natl. Acad. Sci. USA 1974; 71(9):3649-3653.	☐
/A.B./	155	LIZARDI, et al. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet. 1998 Jul;19(3):225-32.	☐
/A.B./	156	LOCKHART, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nature Biotechnology, 14: 1675-1680 (1996).	☐
/A.B./	157	MAG et al. Synthesis and selective cleavage of an oligodeoxynucleotide containing a bridged internucleotide 5'-phosphorothioate linkage. Nucleic Acids Res 19(7):1437-1441 (1991).	☐
/A.B./	158	MAKRIGIOGOS, G. Fluorescent Labeling of Abasic Sites: A Novel Methodology to Detect Closely-Spaced Damage Sites in DNA. Int. J. Radiat. Biol. 1998; 74(1):99-109.	☐
/A.B./	159	MARCHUK, et al. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. 1991 March 11; 19(5): 1154.	☐
/A.B./	160	MARDIS, E. New strategies and emerging technologies for massively parallel sequencing: applications in medical research. Online 2009 April 17. Genome Med. 2009; 1(4): 40. Available at www.ncbi.nlm.nih.gov/pmc/articles/PMC2684661/?tool=pubmed. Accessed 10/22/2009.	☐
/A.B./	161	MARDIS. Next-Generation DNA Sequencing Methods. The Annual Review of Genomics and Human Genetics. 2008; 9:387-402.	☐
/A.B./	162	MARGULIES, et al. Genome sequencing in microfabricated high-density picolitre reactors [abstract]. Nature. 2005 Sep 15; 437 (7057): 376-80. Epub 2005 July 31.	☐

Examiner Signature	/ANGELA M. BERTAGNA/	Date Considered	04/21/2020
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Substitute for form 1449/PTO		Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Application Number	16017340
		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
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NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
/A.B./	163	MASKOS et al. Oligonucleotide hybridisations on glass supports: a novel linker for oligonucleotide synthesis and hybridisation properties of oligonucleotides synthesised in situ. Nucleic Acids Research. 1992;20(7):1679-1684.	☐
/A.B./	164	MAULIK, et al. Novel Non-isotopic Detection of MutY Enzyme-recognized Mismatches in DNA Via Ultrasensitive Detection of Aldehydes. Nucl. Acids. Res. 1999: 27(5):1316-1322.	☐
/A.B./	165	MCCARTHY, et al. Inducible repair of O-alkylated DNA pyrimidines in Escherichia coli. EMBO J. 1984; 3(3):545-50.	☐
/A.B./	166	MCCLURE, et al. Bovine exome sequence analysis and targeted SNP genotyping of recessive fertility defects BH1, HH2, and HH3 reveal a putative causative mutation in SMC2 for HH3. PLoS One. 2014 Mar 25;9(3):e92769. doi: 10.1371/journal.pone.0092769. eCollection 2014.	☐
/A.B./	167	MCHUGH, et al. Novel Regents for Chemical Cleavage at Abasic Sites and UV Photoproducts in DNA. Nucl. Acids. Res. 23(10): 1664-1670.	☐
/A.B./	168	MEIER et al. Peptide Nucleic Acids (PNAs)-Unusual Properties of Nonionic Oligonucleotide Analogues. Angew. Chem. Int. Ed. Engl.31(8):1008-1010 (1992).	☐
/A.B./	169	MEISSNER, et al. Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. Nucleic Acids Res. 2005 Oct 13;33(18):5868-77. Print 2005.	☐
/A.B./	170	METZKER, M. Sequencing technologies - the next generation. Nat Rev Genet. 2010 Jan;11(1):31-46. Epub 2009 Dec 8.	☐
/A.B./	171	MEUZELAAR, et al. MegaPlex PCR: a strategy for multiplex amplification. Nat Methods. 2007 Oct;4(10):835-7. Epub 2007 Sep 16.	☐

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/A.B./	172	MEYER, et al. Parallel tagged sequencing on the 454 platform. Nat Protoc. 2008;3(2):267-78. doi: 10.1038/nprot.2007.520.	☐
/A.B./	173	MEYER, et al. Targeted high-throughput sequencing of tagged nucleic acid samples. Nucleic Acids Res. 2007;35(15):e97.	☐
/A.B./	174	MITCHELL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008 Jul 29;105(30):10513-8.	☐
/A.B./	175	MITRA, et al., In situ localized amplification and contact replication of many individual DNA molecules. Nucleic Acids Research. 1999. 27:(24); e34.	☐
/A.B./	176	MITRA, et al. Oxidative DNA cleavage by the antitumor antibiotic leinamycin and simple 1,2-dithiolan-3-one 1-oxides: Evidence for thiol-dependent conversion of molecular oxygen to DNA-cleaving oxygen radicals mediated by polysulfides. Journal of the American Chemical Society. 1997; Vol.119(48):11691-11692.	☐
/A.B./	177	MIZUGAKI, et al. Preparation of a monoclonal antibody specific for 5-methyl-2'-deoxycytidine and its application for the detection of DNA methylation levels in human peripheral blood cells. Biol Pharm Bull. 1996; 19(12):1537-1540.	☐
/A.B./	178	MOLECULAR PROBE HANDBOOK Section 3.2 obtained from website at: http://www.probes.com/handbook/print/0302.html (Copyright© 1996-2003 by Molecular Probes, Inc.) Visited on August 13, 2003. (18 pages).	☐
/A.B./	179	MULLIS, K.B et al., Eds. (1994). PCR: Polymerase Chain Reaction. Birkhauser: Boston, pp. xv-xvii (Table of Contents).	☐
/A.B./	180	MYLLYKANGAS, et al. Efficient targeted resequencing of human germline and cancer genomes by oligonucleotide-selective sequencing. Nat Biotechnol. 2011 Oct 23;29(11):1024-7. doi: 10.1038/nbt.1996.	☐

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/A.B./	181	NAKAMURA, et al. Highly Sensitive Apurinic/Apyrimidinic site Assay Can Detect Spontaneous and Chemically Induced Depurination Under Physiological Conditions. Cancer Res. 1998; 58:222-225.	☐
/A.B./	182	NAYAK, et al. Functional architecture of T7 RNA polymerase transcription complexes. J. Mol Biol. 2007 August 10; 371(2): 490-500.	☐
/A.B./	183	NEDDERMAN, et al. Cloning and expression of human G/T mismatch-specific thymine-DNA glycosylase. J Biol Chem. 1996; 271(22):12767-74.	☐
/A.B./	184	New England BioLabs Inc. NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®. Available at https://www.neb.com/products/e7420-nebnext-ultra-directional-rna-library-prep-kit-for-illumina . Accessed 06/04/2014.	☐
/A.B./	185	Nextera® Rapid Capture Enrichment Low-Plex Pooling Guidelines. Technical Note: DNA Analysis. 2014. http://www.illumina.com/content/dam/illumina-marketing/documents/products/technotes/technote-nextera-rapid-capture-low-plex-pooling-guidelines.pdf .	☐
/A.B./	186	NEYLON, et al. Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for directed evolution. Nucleic Acids Res. 2004 Feb 27;32(4):1448-59. Print 2004.	☐
/A.B./	187	NG, et al. Targeted capture and massively parallel sequencing of 12 human exomes. Nature. 2009 Sept. 10. 461, 272-276. http://www.nature.com/nature/journal/v461/n7261/full/nature08250.html . Accessed October 6, 2009.	☐
/A.B./	188	NIKOLAEV, et al. Detection of genomic variation by selection of a 9Mb DNA region and high throughput sequencing. PLoS ONE. 2009 Aug. 17. 4(8): e6659.	☐

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		Art Unit	Not Yet Assigned
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/A.B./	189	Notice of allowance dated 07/28/2015 for US Application No. 13/643,056.	☐
/A.B./	190	NUGEN, Inc. Ovation Biotin RNA Amplification and Labeling System User Guide. Catalog #2300-12. Published 2004.	☐
/A.B./	191	NUGEN, Inc. Technical Report #1. The Ovation Biotin System Validation for Use with Affymetrix GeneChip Arrays. Published 2004.	☐
/A.B./	192	OCHMAN, et al. Genetic applications of an inverse polymerase chain reaction. Genetics. 1988 Nov;120(3):621-3.	☐
/A.B./	193	Office action dated 01/16/2013 for US Application No. 12/938,112.	☐
/A.B./	194	Office action dated 02/08/2012 for EP Application No. 07810169.8.	☐
/A.B./	195	Office action dated 02/17/2011 for US Application No. 12/305,633.	☐
/A.B./	196	Office action dated 02/28/2013 for US Application No. 13/156,294.	☐
/A.B./	197	Office action dated 03/01/2010 for US Application No. 10/441,663.	☐
/A.B./	198	Office Action dated 03/03/2017 for US Application No. 15/154,414.	☐
/A.B./	199	Office action dated 03/07/2007 for US Application No. 10/441,663.	☐
/A.B./	200	Office action dated 03/09/2015 for CN Application No. 201380006942.4.	☐
/A.B./	201	Office Action dated 03/15/2017 for US Application No. 14/390,012.	☐

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		Filing Date	06-25-2018		
		First Named Inventor	AMORESE, Doug		
		Art Unit	Not Yet Assigned		
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/A.B./	202	Office action dated 04/03/2015 for CN Application No. 2012800608251.	☐
/A.B./	203	Office action dated 04/16/2014 for US Application No. 13/239,226.	☐
/A.B./	204	Office action dated 05/16/2011 for US Application No. 11/948,784.	☐
/A.B./	205	Office action dated 05/25/2006 for US Application No. 10/441,663.	☐
/A.B./	206	Office action dated 06/06/2012 for US Application No. 10/441,663.	☐
/A.B./	207	Office action dated 06/19/2013 for US Application No. 12/855,611.	☐
/A.B./	208	Office action dated 06/27/2013 for US Application No. 12/938,112.	☐
/A.B./	209	Office action dated 06/30/2008 for US Application No. 11/026,280.	☐
/A.B./	210	Office action dated 07/05/2007 for US Application No. 10/441,663.	☐
/A.B./	211	Office action dated 07/08/2009 for US Application No. 10/441,663.	☐
/A.B./	212	Office action dated 07/09/2015 for US Application No. 14/211,261.	☐
/A.B./	213	Office action dated 07/13/2007 for US Application No. 11/026,280.	☐
/A.B./	214	Office action dated 07/15/2008 for US Application No. 10/441,663.	☐
/A.B./	215	Office action dated 07/15/2015 for US Application No. 13/938,059.	☐
/A.B./	216	Office action dated 07/21/2016 for US Application No. 14/634,326.	☐

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/A.B/	221	Office action dated 09/18/2006 for US Application No. 10/441,663.	☐
/A.B/	222	Office action dated 09/24/2009 for US Application No. 10/441,663.	☐
/A.B/	223	Office action dated 09/24/2014 for US Application No. 13/239,226.	☐
/A.B/	224	Office action dated 10/09/2013 for US Application No. 12/938,112.	☐
/A.B/	225	Office action dated 10/14/2010 for US Application No. 11/948,784.	☐
/A.B/	226	Office Action dated 10-31-2016 for European Application 13806978.6.	☐
/A.B/	227	Office action dated 11/04/2015 for US Application No. 14/030,761.	☐
/A.B/	228	Office action dated 11/07/2012 for US Application No. 13/411,170.	☐
/A.B/	229	Office action dated 11/13/2012 for US Application No. 12/855,611.	☐
/A.B/	230	Office action dated 12/05/2008 for US Application No. 10/441,663.	☐
/A.B/	231	Office action dated 12/17/2007 for US Application No. 10/441,663.	☐

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/A.B/	232	O'SHANNESY, et al. Immobilization of Glycoconjugates by Their Oligosaccharides: Use of Hydrazido-Derivatized Matrices. Anal. Biochem. 1990; 191:1-8.	☐
/A.B/	233	OKOU, et al. Microarray-based genomic selection for high-throughput resequencing. Nat Methods. 2007 Nov;4(11):907-9. Epub 2007 Oct 14.	☐
/A.B/	234	OLIVARIUS, et al. High-throughput verification of transcriptional starting sites by Deep-RACE. Biotechniques. 2009 Feb;46(2):130-2. doi: 10.2144/000113066.	☐
/A.B/	235	OLSON, M. Enrichment of super-sized resequencing targets from the human genome. Nat Methods. 2007 Nov;4(11):891-2.	☐
/A.B/	236	Openwetware. Directional-RNAseq Prep. Available at http://openwetware.org/wiki/Directional-RNAseq_Prep . Accessed 06/04/2014.	☐
/A.B/	237	OYOLA, et al. Efficient Depletion of Host DNA Contamination in Malaria Clinical Sequencing. J. Clin. Microbiol. March 2013; 51(3):745-751.	☐
/A.B/	238	PABINGER, et al. A survey of tools for variant analysis of next-generation genome sequencing data. Brief Bioinform. 2014 Mar;15(2):256-78. doi: 10.1093/bib/bbs086. Epub 2013 Jan 21.	☐
/A.B/	239	PANG, et al. Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA. Molecular and Cellular Probes. 1992; 6:251-256.	☐
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		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
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/A.B./	241	PARKHOMCHUK, et al. Transcriptome analysis by strand-specific sequencing of complementary DNA. Nucleic Acids Res. 2009 Oct;37(18):e123.	☐
/A.B./	242	PAUWELS et al. Biological activity of new 2-5A analogues. Chemica Scripta 26:141-149 (1986).	☐
/A.B./	243	PEASE, et al. A rapid, directional RNA-seq library preparation workflow for Illumina [reg] sequencing. Nature Methods. 2012; 9, no. 3.	☐
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/A.B./	246	PEI, et al. Site-specific cleavage of duplex DNA by semisynthetic nuclease via triple-helix formation. Pro. Natl. Acad. Sci. USA. 1990 Dec. 87: 9858-9862.	☐
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/A.B./	251	PRASHAR, et al. Analysis of differential gene expression by display of 3' end restriction fragments of cDNAs. Proc Natl Acad Sci U S A. 1996 Jan 23;93(2):659-63.	☐
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/A.B./	268	SCHENA, et al. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc Natl. Acad. Sci. USA Biochemistry. 1996; 93:10614-10619.	☐
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/A.B./	270	SCHMID, et al. Chic and chec: genomic mapping of chromatin proteins. Molecular Cell. 2004. 16, no1, pp. 147-157. (abstract).	☐
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/A.B./	277	Singapore written opinion dated 03/17/2015 for SG Application No. 11201401628W.	☐
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/A.B./	280	SONI, et al. Progress toward ultrafast DNA sequencing using solid-state nanopores. Clin Chem. 2007 Nov;53(11):1996-2001. Epub 2007 Sep 21.	☐
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/A.B./	284	STEPHPENS, et al. Automating sequence-based detection and genotyping of SNPs from diploid samples. Nat Genet. 2006 Mar;38(3):375-81. Epub 2006 Feb 19.	☐
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/A.B./	288	SUGIYAMA, et al. Chemistry of thermal degradation of abasic sites in DNA. Mechanistic investigation on thermal DNA stand cleavage of alkylated DNA. Chem. Res. Toxicol. 1994; 1:673-683.	☐
/A.B./	289	SUMMERER, D. Enabling technologies of genomic-scale sequence enrichment for targeted high-throughput sequencing. Genomics. 2009 Dec;94(6):363-8. (abstract).	☐

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/A.B/	290	SUMMERER, et al. Microarray-based muticycle-enrichment of genomic subsets for targeted next-generation sequencing. Accepted 2009 June 18. Available at www.ncbi.nlm.nih.gov/pubmed/19638418. Accessed 10/6/2009.	☐
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/A.B/	298	VARKONYI-GASIC, et al. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods. 2007 Oct 12;3:12.	☐

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/A.B/	299	VARLEY, et al. Nested Patch PCR enables highly multiplexed mutation discovery in candidate genes. Genome Res. 2008 Nov;18(11):1844-50. doi: 10.1101/gr.078204.108. Epub 2008 Oct 10.	☐
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If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

Substitute for form 1449/PTO		Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Application Number	16017340
		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
Sheet	58	of	61

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
/A.B./	309	WU, et al. Phasing Amplicon Sequencing For Robust Microbial Community Analysis. I-2630. 2014. http://www.asmonlineeducation.com/php/asm2014abstracts/data/papers/I-2630.htm .	☐
/A.B./	310	XIAO, et al. Sequential amplification of flanking sequences by Y-shaped adaptor dependent extension using multiple templates. Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao (Journal Of Plant Physiology and Molecular Biology). 2007 Feb;33(1):85-90.	☐
/A.B./	311	YOUNG, et al. A new strategy for genome assembly using short sequence reads and reduced representation libraries. Genome Res. 2010 Feb;20(2):249-56. doi: 10.1101/gr.097956.109.	☐
/A.B./	312	ZALIPSKY, S. Functionalized Poly(ethylene glycol) for Preparation of Biologically Relevant Congugates. Bioconjugate Chem. 1995; 6:150-165.	☐
/A.B./	313	ZANG, et al. DNA alkylation by leinamycin can be triggered by cyanide and phosphines. Bioorg Med Chem Lett. 2001 Jun 18;11(12):1511-5.	☐
/A.B./	314	ZHANG, et al. Multiplex sequencing on the SOLID platform with 10, 16, or 96 barcodes. 2009 Life technologies. www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_065528.pdf .	☐
/A.B./	315	ZHENG, et al. Titration-free 454 sequencing using Y adapters. Nat Protoc. 2011 Aug 18;6(9):1367-76. doi: 10.1038/nprot.2011.369.	☐
/A.B./	316	ZHU, et al. Overexpression of 5-methylcytosine DNA glycosylase in human embryonic kidney cells EcR293 demethylates the promoter of a hormone-regulated reporter gene. Proc Natl Acad Sci USA. 2001; 98(9):5031-6.	☐
/A.B./	317	ZHU, et al. 5-Methylcytosine DNA glycosylase activity is also present in the human MBD4 (G/T mismatch glycosylase) and in a related avian sequence. Nucleic Acids Res. 2000; 28(21):4157-65.	☐

Examiner Signature	/ANGELA M. BERTAGNA/	Date Considered	04/21/2020
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Complete if Known			
		Application Number	16017340		
		Filing Date	06-25-2018		
		First Named Inventor	AMORESE; Doug		
		Art Unit	Not Yet Assigned		
		Examiner Name	Not Yet Assigned		
Sheet	59	of	61	Attorney Docket Number	25115-755.302

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
/A.B./	318	ZHULIDOV, et al. Simple cDNA normalization using kamchatka crab duplex=specific nuclease. Nucleic Acids Research. Online 2004 Feb. 18. 32:3 e37.	☐
/A.B./	319	ZILLER, et al. Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. PLoS Genet. 2011 Dec;7(12):e1002389. doi: 10.1371/journal.pgen.1002389. Epub 2011 Dec 8.	☐

Examiner Signature	/ANGELA M. BERTAGNA/	Date Considered	04/21/2020
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

Substitute for form 1449/PTO		Complete if Known	
		Application Number	16017340
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Filing Date	06-25-2018
		First Named Inventor	AMORESE; Doug
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
		Attorney Docket Number	25115-755.302
Sheet	60	of	61

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

- ☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

- ☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

- ☐ See attached certification statement.
- ☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.
- ☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jeffrey Seidel/	Date (YYYY-MM-DD)	2018-12-04
Name/Print	Jeffrey Seidel	Registration Number	61,063

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

<i>Search Notes</i> 	Application/Control No. 16/017,340	Applicant(s)/Patent Under Reexamination AMORESE et al.
	Examiner Angela Bertagna	Art Unit 1637

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
searched all inventors by name in DAV	04/25/2020	amb
EAST search history attached (text & inventor name)	04/25/2020	amb
reviewed parent cases	04/25/2020	amb

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

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PATENT
Attorney Docket No. 25115-755.301

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application:

Inventor: Doug Amorese *et al.*

Application No.: 15/471,785

Filed: March 28, 2017

Title: COMPOSITIONS AND METHODS
FOR TARGETED NUCLEIC ACID
SEQUENCE ENRICHMENT AND
HIGH EFFICIENCY LIBRARY
REGENERATION

Confirmation No.: 8042

Examiner: Angela Bertagna

Group Art Unit: 1637

Customer No. 12754

DECLARATION OF ANDREW BROOKS
UNDER 37 C.F.R. §1.132

M/S Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF ANDREW BROOKS

Commissioner:

I, Andrew I. Brooks, declare as follows:

I. QUALIFICATIONS

1. My name is Andrew I. Brooks.
2. I am the Chief Operating Officer at RUCDR Infinite Biologics at Rutgers. RUCDR Infinite Biologics provides a wide array of genotyping, gene expression, and microarray services, and provides consultation for experimental design, platform and microarray selection, as well as bioinformatics support. RUCDR Infinite Biologics offers massively parallel next-generation sequencing on the latest platforms from Ion Torrent and Illumina. Comprehensive service bundles offered by RUCDR Infinite Biologics include nucleic acid extraction, integrated sample quality control, and fully automated construction of sequencing libraries.
3. I am also President and Founder of Brooks Biomedical Consulting, BBC LLC, which provides consulting services for personalized medicine, animal model development, gene-based therapeutics, molecular diagnostics, and enterprise level biobanking.

4. I am an Associate Professor of Genetics at Rutgers, The State University of New Jersey and am Director of the Bionomics Research & Technology Center at the Environmental & Occupational Health Sciences Institute at Rutgers University.
5. Previously, I served as the Director of the Functional Genomics Center at the Department of Environmental Medicine at the Aab Institute for Biomedical Science at the University of Rochester Medical School and Director of the Academic Medicine Development Company (AMDeC) Microarray Resource Center (MRC) at the University of Rochester Medical Center.
6. I also previously served as Director of the University of Rochester Medical Center Core Facilities, which provided microarray core services and nucleic acid core services, and was a Director of the Microarray Analysis Core Facility at the National Institute of Aging Nathan Shock Center for Excellence.
7. I hold a B.S. in Animal Science from Cornell University, and a M.S. and Ph.D. in Molecular Medicine and Gene Therapy from University of Rochester School of Medicine.
8. I am a member of the Board of Directors for several companies including NuGEN Technologies and previously was a member of the Scientific and Commercial Advisory Board at NuGEN Technologies.
9. I am a member of the American Society for Human Genetics, American Society for Gene Therapy, The Association of Biomolecular Resource Facilities, and Society for Neuroscience among others. I serve or have served on several committees including NIEHS CEED Center Internal Advisory Board, EOHSI Directors Cabinet, University of Rochester Scientific Advisory Board (SAC), and Microarray Research Group at the Association of Biomolecular Resource Facilities. I am a peer reviewer for several journals, most notably Nature Genetics, Nature Medicine, and Nucleic Acid Research.
10. I have published numerous peer-reviewed manuscripts and presented at various international conferences. My research interest has focused on the development and implementation of cutting edge molecular based technologies for nucleic acid and protein analyses.
11. A copy of my *curriculum vitae* is attached as Appendix A.

II. SCOPE OF REVIEW

12. I have read the pending claims of the above-referenced application, and am familiar with the Office Action mailed on August 18, 2017. I understand that the United States Patent and Trademark Office ("Patent Office") has rejected the pending claims of U.S. Patent Application No. 15/471,785 ("785 application") under pre-AIA U.S.C. § 103(a) over U.S. Publication No. 2005/0142577 ("Jones") in view of Tucker et al. (The American Journal of Human Genetics (2009) 85: 142-154; "Tucker"), and further in view of U.S. Patent No. 7,741,463 ("Gormley") or Rothberg et al. (Nature (2011) 475: 348-352; "Rothberg").

III. SKEPTICISM AND UNEXPECTED RESULTS

13. NuGEN approached me in 2012 and encouraged me to use methods claimed in the '785 application to generate enriched sequences for sequencing on a massively parallel sequencing platform.

14. Based on my experience in the field, I was skeptical that the claimed methods would provide efficient sequencing data. Because the claimed methods involve annealing and extending a target-specific primer to enrich a sequence of interest (rather than, for example, annealing a forward target-specific and reverse target-specific primer pair at a sequence of interest and performing a polymerase chain reaction with the forward target-specific and reverse target-specific primer), I thought the technology would provide poor target specificity that is not consistent with enrichment worthy of high quality sequencing data. I thought that the target-specific primers would bind to off-target sites at a relatively high rate, leading to non-specific enriched nucleic acid sequences that would flood the sequencing system and confound sequence analysis. I thought that in order to generate enriched sequences that would provide highly specific sequencing results, one would want to amplify a sequence of interest with, for example, a primer pair including a forward target-specific primer and reverse target-specific primer in a polymerase chain reaction using the forward target-specific primer and reverse target-specific primer for each sequence of interest.

15. The lack of specificity that I expected would have been a significant detriment. For example, inability to achieve high specificity would result in significant waste associated with performing massively parallel sequencing runs. Additionally, I felt that significant off-target activity would affect the ability to reliably create a uniform distribution of on target sequences leading not only to an

incomplete (or less complex) library but one that had such an uneven distribution of targets that our ability to make quantitative analyses would be severely impaired.

16. In view of my skepticism, I observed unexpected results when enriched nucleic acid sequences generated using the claimed methods were sequenced by massively parallel sequencing. Based on my skepticism, using the claimed methods I expected to observe about fifty percent of sequences of interest in the sequencing results. We actually observed greater than ninety percent of on-target sequences of interest in the sequencing results. Based on my knowledge and experience in this field, I did not expect and would not have predicted this level of specificity. The results were surprising to me. I would not have thought to utilize massively parallel sequencing in this manner and did so because I was asked by NuGEN to perform the method.

17. Jones describes an enrichment method employing a target-specific primer for generation of amplification products for analysis with microarrays. In contrast to the claimed methods, microarray analysis relies on hybridization of the target sequence of the amplified product to a unique complementary probe on the microarray. If a target-specific primer anneals to the target sequence, the resulting on-target amplified product will hybridize to the unique complementary probe on the microarray. On the other hand, if the target-specific primer anneals to off-target sequences, there would be no equivalent complementary probe on the microarray to hybridize to the resulting off-target amplified product. Thus, unlike on a massively parallel sequencing platform, the off-target product that may be produced would not confound analysis since the noise from off-target sequences would be minimal or absent.

IV. CONCLUDING STATEMENTS

18. I declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and 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 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

19. While I am being compensated for my time in connection with this declaration, that compensation does not depend in any way on the outcome of the prosecution of the '785 patent application.

Dated: February , 2018

By:

Andrew I. Brooks

15 Feb 2018

Appendix A

Andrew I. Brooks, Ph.D.

CURRICULUM VITAE

NAME: Andrew I. Brooks

ADDRESS: Office: Environmental & Occupational Health Sciences Institute
Bionomics Research & Technology Center
170 Frelinghuysen Road - Room 236H
Piscataway, NJ 08854

Home: 3 Hanover Square, PH23A
New York, New York 10004

TELEPHONE: Office: (848) 445-0225

FACSIMILE: (732) 445-0152

EMAIL: brooks@dls.rutgers.edu

CITIZENSHIP: USA

EDUCATION

Cornell University, Ithaca, NY
Animal Science

B.S. 1991

Albert Einstein College of Medicine, Bronx, NY
Neuroscience

Ph.D. candidate 1994-1995

University of Rochester School of Medicine, Rochester, NY 14642
Department of Microbiology and Immunology
Molecular Medicine & Gene Therapy

M.S. 1998

University of Rochester School of Medicine, Rochester, NY 14642
Department of Microbiology and Immunology
Molecular Medicine & Gene Therapy

Ph.D. 1999

Appendix A

Andrew I. Brooks, Ph.D.

ACADEMIC APPOINTMENTS

2009 – Present	Chief Operating Officer RUCDR Infinite Biologics Rutgers University - Rutgers Medical School Associate Professor of Genetics Associate Director for Technology Development Rutgers, The State University of New Jersey
2012 – Present	Co-Founding Director BioProcessing Solutions Alliance BioStorage Technologies Indianapolis, Indiana
2006- Present	Director and Founder, Harlan GenScreen™ Laboratory Harlan Laboratories Indianapolis, Indiana
2005 – 2009	Associate Professor of Genetics Associate Director for Technology Development Rutgers University Cell & DNA Repository Rutgers, The State University of New Jersey
2005 – Present	Director, Bionomics Research & Technology Center Associate Professor of Environmental Medicine Environmental & Occupational Health Sciences Institute University of Medicine & Dentistry of New Jersey
2004 – Present	President and Founder, Brooks Biomedical Consulting, BBC LLC Consulting services for Personalized Medicine, Animal Model Development, Gene-based Therapeutics, Molecular Diagnostics
May 2004 – Present	Adjunct Associate Professor Rochester Institute of Technology School of Computer Science and Bioinformatics
May 2003 – Present	Food and Drug Administration (FDA), Special appointment for the Pharmacology Toxicology Subcommittee of the Advisory Committee for Pharmaceutical Science
September 2001-2005	Director, AMDeC Microarray Resource Center, Host Institution: University of Rochester Medical Center
January 2001-2005	Assistant Professor Director, Functional Genomics Center Department of Environmental Medicine

Appendix A

Andrew I. Brooks, Ph.D.

January 2001-2005	Aab Institute for Biomedical Science University of Rochester Medical School Director, University of Rochester Medical Center Core Facilities Microarray Core Services Nucleic Acid Core Services Proteomics Core Services Bioinformatics Training Services
January 2001- June 2002	Director, Microarray Analysis Core Facility, NIA Nathan Shock Center for Excellence
September 1999-December 2000	Research Assistant Professor Director, University Microarray Core Facility Center for Aging & Development Division of Molecular Medicine & Gene Therapy Aab Institute for Biomedical Science University of Rochester Medical School

AWARDS AND HONORS

- 1990 Scholarship from Dialogue House Inc. for work in cancer research
- 1997 Excellence in Graduate Research Award Division of Molecular Medicine & Gene Therapy
- 1998 American Society for Gene Therapy Young Investigator Award
- 2000 Co-chair Neuromuscular Session 3rd Annual American Society for Gene Therapy Annual Meeting
- 2000 University of Rochester Fenn Award for Distinguished Ph.D. Thesis
- 2002 Scientific Director, Convergence Biotechnology Conference
- 2002 Chair, Microarray Research Group (MARG), Association of Biomedical Resource Facilities
- 2002 Co-Chair Neurology Session 5th Annual American Society for Gene Therapy Annual Meeting
- 2003 Executive Board Member, UNYCoR
- 2003 Scientific and Commercial Advisory Board, NuGen Technologies
- 2003 Chair, Microarray Research Group (MARG), Association of Biomedical Resource Facilities
- 2003 Scientific Advisory Board, Biocept Inc.
- 2005 Chief Scientific Officer, URMCO Office of Corporate Alliance (OCA)
- 2005 Co-Director, Planning Committee, Expanded Regional Translational Research Center
- 2015 American Association for Clinical Chemistry (AACC)'s Outstanding Speaker Award

CORPORATE PROGRAMS

- 2000 Affymetrix Inc.; Academic Center of Excellence
- 2001 Socratech LLC; Microarray/Gene Expression Services
- 2001 Corning Inc.; Life Sciences Division, Technology Development Program
- 2001 STS Biopolymers; High Technology of Rochester Technology Development Contract
- 2003 Early Access Program, NuGen Technologies
- 2014 NuGEN Center of Excellence Director
- 2014 Raindance Center of Excellence Director

BOARD SEATS

- BioDX Technologies, San Diego CA
- NuGen Technologies, San Francisco CA

Appendix A

Andrew I. Brooks, Ph.D.

GenScreen, Indianapolis IN
Angiocrine, San Diego CA
BioProcessing Solutions Alliance, Indianapolis IN
iSpecimen, Boston MA

PROFESSIONAL MEMBERSHIP

Society for Neuroscience (SFN)
American Society for Gene Therapy(ASGT)
The Association of Biomolecular Resource Facilities (ABRG)
Academic Medicine Development Company (AMDeC)
Upstate New York Coalition of Biomedical Research (UNYCoR)
American Association for Laboratory Animal Science (AALAS)
American Society for Human Genetics (ASHG)
International Society for Biological and Environmental Repositories (ISBER)
Society for Laboratory Automation and Screening (SLAS)

TEACHING EXPERIENCE

Medical Microbiology (Teaching Assistant)
Cellular Neuroscience (Lecturer)
Oral Biology Seminar Series (Lecturer)
Fundamentals of Computational Biology (Lecturer)
Genes Development and Disease (GEN 508) (Lecturer)
Introductory Toxicology (TOX 508) (Lecturer)
Introduction to Basic Concepts of Molecular Biology & Bioinformatics (Lecturer)
Molecular Epidemiology Series (Lecturer)
Toxicogenomics/Proteomics (TOX 521) (Lecturer)
Functional Genomics of Neuronal and Neurodegeneration: A Case Study (Lecturer)

COMMITTEE SERVICE

2000 / 2002	Abstract peer review committee (American Society for Gene Therapy; by invitation)
2000 – 2005	University of Rochester Biohazard Committee
2001 – 2005	University of Rochester Biomedical Research Capital Facility Planning Committee
2001 – 2013	AMDeC Functional Genomics Advisory Committee
2001 – 2006	Microarray Research Group; Association of Biomolecular Resource Facilities
2003 – 2005	Rochester Center for Computational Systems Biology (RCCSB)
2004 – 2005	University of Rochester Office of Corporate Alliances Planning Committee
2004 – 2005	University of Rochester Wyeth Building Planning Committee
2005 – 2005	University of Rochester Intellectual Property Advisory Committee
2005 – 2008	University of Rochester Scientific Advisory Board (SAC)
2005 – 2008	External Scientific Advisory Bd-Regional Translational Research Center (NYU-HHC-RTRC)
2005 – Present	Rutgers, The State University of New Jersey Joint Graduate Program in Toxicology Committee
2005 – Present	EOHSI Directors Cabinet
2005 – Present	NIEHS CEED Center Internal Advisory Board

PEER REVIEWER

Journals

Neurobiology of Aging (Editor in chief: Paul D. Coleman, PhD)

Appendix A

Andrew I. Brooks, Ph.D.

Leukemia Research (Editor in chief: John M. Bennett, MD)

Nucleic Acid Research

European Journal of Neuroscience

Microbiology UK

Nature Genetics

Nature Medicine

The Journal of Gene Medicine

Meetings

American Society for Gene Therapy

NERLSCD-Regional Core Directors Meeting

Grants

Alzheimer's Association

NIEHS

Mehta Foundation

Marsden Foundation

Northwest Cancer Research Fund

Environmental Protection Agency

American Federation of Aging Research (AFAR)

NINDS Microarray Consortium

AAAS Applications Review

NIAAA Applications Review

INVITED PRESENTATIONS: 2005 – Present

2005 ABRF Annual Meeting in Atlanta, Georgia

2005 SOT-ToxExpo in New Orleans, Louisiana

2005 CHI Quantitative PCR Conference in La Jolla, California

2005 SUNY Upstate Translational Research Conference (Panel Speaker)

2005 Hills Pet Nutrition (Speaker)

2005 IBC 12th Annual Chips to Hits International Conference

2006 Society of Toxicology Annual Meeting in San Diego, California (Panel Speaker)

2006 CHI, QPCR Conference in San Diego, California (Panel Speaker)

2006 CHI QPCR Short Course in Boston, Massachusetts

2006 International Seminar for Toxicogenomics (Pavia, Italy)

2007 NuGEN Scientific Advisory Board Meeting

2007 ABRF 2007 Annual Meeting in Tampa, Florida (Conference Co-Chair)

2007 International Society for Biological and Environmental Repositories in Singapore (Panel Speaker)

2007 CHI Point of Care Diagnostics in Washington, DC (Panel Speaker)

2007 NIH Workshop in Rockville, MD (Speaker)

2007 World Congress on Psychiatric Genetics XV Conference

2008 ABRF 2008 Annual Meeting in Salt Lake City, UT (Panel Speaker)

2008 CHI QPCR Conference in San Diego, CA (Speaker)

2008 International Society for Prenatal Diagnosis (Speaker)

2008 Institute for International Research Biorepositories Conference in Philadelphia, PA (Speaker)

2008 CHI Data Driven Discovery Conference in Providence, RI

2009 Next Gen Sequencing, San Diego, CA

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- 2009 International Society for Biological and Environmental Repositories in Portland, OR
- 2009 Institute for International Research Biorepositories Conference, Philadelphia, PA
- 2009 American Society of Human Genetics 59th Annual Meeting in Honolulu, HI
- 2009 Biobanking at Cornell University, Ithaca, NY
- 2009 Science of Biobanking in Philadelphia, PA
- 2010 Lab Automation 2010 in Palm Springs, CA
- 2010 STARLIMS Forum in Orlando, FL
- 2010 3rd Annual Biospecimen Research Network Symposium in Bethesda, MD
- 2010 International Society for Biological and Environmental Repositories in Rotterdam, Netherlands
- 2010 CHI Beyond Sequencing in San Francisco, CA
- 2010 Institute for International Research Biorepositories Conference in Boston, MA
- 2010 World Congress on Psychiatric Genetics in Athens, Greece
- 2010 American Association for Laboratory Animal Science National Meeting in Atlanta, GA
- 2010 Northeast Regional Life Sciences Core Directors Biorepositories & Biobanking Conference in Worcester, MA
- 2010 Trends in Science and Technology Relevant to the Biological Weapons Convention in Beijing, China
- 2010 American Society of Human Genetics Annual Meeting in Washington, DC
- 2010 World Congress of Gene-2010 in Foshan, China
- 2010 CHI Biobanking Meeting in Providence, RI
- 2011 Lab Automation 2011 in Palm Springs, CA
- 2011 Global Sample Management Asia Event in San Francisco, CA
- 2011 American Society of Human Genetics Annual Meeting in Montreal, Canada
- 2011 Cryo2011 Event at Oregon State University in Corvallis, OR
- 2012 Society of Laboratory Automation and Screening in San Diego, CA
- 2012 Advances in Genome Biology & Technology on Marco Island, FL
- 2012 5th Annual Biospecimen Research Network Symposium in Bethesda, MD
- 2012 International Society for Biological and Environmental Repositories in Vancouver, Canada
- 2012 Association of Biomolecular Resource Facilities Conference in Orlando, FL
- 2012 European Lab Automation in Hamburg, Germany
- 2012 Genomics Research Europe in Frankfurt, Germany
- 2012 Institute for International Research Biorepositories Clinical Business Expo in Boston, MA
- 2012 Leaders in BioBanking Congress in Chapel Hill, NC
- 2012 Digital PCR - Applications and Advances in San Diego, CA
- 2012 American Society of Human Genetics in San Francisco, CA
- 2013 Society of Laboratory Automation and Screening in Orlando, FL
- 2013 Human Genome Meeting in Singapore
- 2013 BioCor, University of Minnesota
- 2013 Biobanking UK Conference in London, UK
- 2013 European, Middle Eastern & African Society for Biopreservation & BioBanking in Verona, Italy
- 2013 Hands On Biobanks, Amsterdam, Netherlands
- 2014 Ninth Annual Biomarkers Congress in Manchester, UK
- 2014 European, Middle Eastern & African Society for Biopreservation & BioBanking LIMS Workshop in Milan, Italy
- 2014 Association of Biomolecular Resource Facilities Conference in Albuquerque, NM
- 2014 American Association for Cancer Research in San Diego, CA
- 2014 International Society for Biological and Environmental Repositories in Orlando, FL
- 2014 7th Annual Biorepositories & Sample Management Conference in Boston, MA

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2014 Leaders in BioBanking Congress in Seattle, WA
2014 North American Saliva Symposium, Boston, MA
2014 NBDA Convergence Conference, Scottsdale, AZ
2015 Qatar Biobank Launch Conference, Al Gharafa, Ar-Rayyan, Qatar
2015 CHI PCR for Molecular Medicine conference, San Francisco, CA
2016 Society of Laboratory Automation and Screening in San Diego, CA
2016 Molecular Med Tri-Con in San Francisco, CA
2016 National Biobank Conference in Shanghai, China
2016 International Society for Biological and Environmental Repositories in Berlin, Germany
2016 Genetic Toxicology Association Meeting in Newark, DE
2016 European Society Human Genetics in Barcelona, Spain
2016 2016 University-Industry Interaction Conference in Amsterdam, The Netherlands
2016 Liquid Biopsy Summit in San Francisco, CA
2016 Leaders in Biobanking Congress in Baltimore, MD
2016 Annual Biorepositories & Sample Management Conference in Boston, MA
2016 World Life Science Conference in Beijing, China
2017 National Biobank Conference in Shanghai, China
2017 Molecular Med Tri-Con, San Francisco, CA
2017 The Impact of Biobanking in Qatar - Precision Medicine Initiative in Doha, Qatar
2017 International Society for Biological and Environmental Repositories in Toronto, Canada
2017 European, Middle Eastern & African Society for Biopreservation in Copenhagen, Denmark
2017 American Society of Human Genetics Annual Meeting in Orlando, FL
2017 Leaders in Biobanking Congress in Nashville, TN
2017 6th Annual Biobank China and International Symposium of Precision Medicine in Changsha, China

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AMENDMENTS TO THE CLAIMS

Please amend the claims as follows:

Listing of claims

1. - 50. (Cancelled)

51. (Currently Amended) A method for sequencing an enriched nucleic acid sequence of interest, the method comprising:

- a) annealing one or more oligonucleotides in solution in a reaction mixture to the nucleic acid sequence of interest in a nucleic acid fragment, wherein the reaction mixture comprises a plurality of nucleic acid fragments, wherein the nucleic acid fragment comprising the nucleic acid sequence of interest comprises a first adaptor sequence, wherein the one or more oligonucleotides comprise a 3' portion with at least 10 bases designed to be complementary to the nucleic acid sequence of interest and a 5' tail portion comprising a second adaptor sequence that is non-complementary to the nucleic acid sequence of interest;
- b) extending the one or more oligonucleotides annealed to the nucleic acid sequence of interest in the nucleic acid fragment comprising the first adaptor sequence with a polymerase, in the reaction mixture, thereby generating one or more oligonucleotide extension products comprising sequence complementary to the first adaptor sequence at a first end, sequence complementary to the nucleic acid sequence of interest, and the second adaptor sequence at a second end;
- c) amplifying the one or more oligonucleotide extension products, in the reaction mixture, using a first primer that anneals to the complement of the first adaptor sequence and a second primer that anneals at its 3' end to a complement of the second adaptor sequence, thereby enriching the nucleic acid sequence of interest, [[there]]by generating amplified products comprising [[an]]the enriched nucleic acid sequence of interest; and
- d) sequencing the amplified products comprising the enriched nucleic acid sequence of interest on a massively parallel sequencing platform.

52. (Previously Presented) The method of claim 51, wherein the nucleic acid sequence of interest comprises genomic DNA.

53. (Previously Presented) The method of claim 51, wherein the nucleic acid sequence of interest comprises cDNA.
54. (Previously Presented) The method of claim 51, wherein the nucleic acid sequence of interest comprises RNA.
55. (Previously Presented) The method of claim 51, wherein the first adaptor sequence and the second adaptor sequence are distinct from each other.
56. (Currently Amended) The method of claim 51, wherein the plurality of nucleic acid fragments comprises the first adaptor sequence, and wherein the first adaptor sequence is common to ~~each of~~ the plurality of nucleic acid fragments.
57. (Currently Amended) The method of claim 51, wherein the one or more oligonucleotides comprises more than one oligonucleotide, wherein the second adaptor sequence is common to the more than one oligonucleotide.
58. (Currently Amended) The method of claim 51, wherein the first adaptor sequence and/or the second adaptor sequence comprise(s) a barcode sequence.
59. (Currently Amended) The method of claim 51, further comprising denaturing double-stranded nucleic acid fragments prior to step a), ~~thereby generating the nucleic acid fragment comprising the first adaptor sequence.~~
60. (Previously Presented) The method of claim 51, wherein the polymerase is a DNA polymerase.
61. (Currently Amended) The method of claim 51, wherein the sequencing comprises the use of four labeled reversible terminators.
62. (Previously Presented) The method of claim 51, wherein the sequencing comprises semiconductor sequencing.
63. (Previously Presented) The method of claim 51, wherein the sequencing comprises nanopore sequencing.

64. (Currently Amended) The method of claim 51, wherein each of the plurality of nucleic acid fragments comprises the first adaptor sequence at a 5' end ~~of each of the plurality of nucleic acid fragments~~.
65. (Previously Presented) The method of claim 51, further comprising fragmenting nucleic acids prior to step a), thereby generating the plurality of nucleic acid fragments.
66. (Currently Amended) The method of claim 51, further comprising ~~appending~~ ligating the first adaptor sequence to a nucleic acid fragment in the plurality of nucleic acid fragments prior to step a), thereby generating the nucleic acid fragment comprising the first adaptor sequence.
67. (Cancelled)
68. (Previously Presented) The method of claim 51, wherein the amplified products comprise a 3' end with sequence complementary to a sequence on a surface.
69. (Previously Presented) The method of claim 68, further comprising annealing a strand of the products of the amplifying to the sequence on the surface using the 3' end with sequence complementary to the sequence on the surface prior to step d).
70. (Previously Presented) The method of claim 68, wherein the surface is a surface of a flow cell.

REMARKS

Claims 51-70 were pending in the subject application. By this Amendment, Applicant has cancelled claim 67 and amended claims 51, 56-59, 64, and 66. The amendments to claims 51, 56-59, 64, and 66 were merely for the purposes of clarity and include no new matter. Upon entry of this Amendment, claims 51-66 and 68-70 will be pending with claim 51 as the sole independent claim.

Objection to the Specification

Page 3 of the non-final Office Action states that the specification is objected to because the continuity data information in the first paragraph should be updated to indicated that prior-filed Application Serial No. 15/471,785 issued as U.S. Patent No. 10,036,012.

Applicant has amendment to the specification to update the continuity data information in paragraph [0001] of the specification by stating that prior-filed Application Serial No. 15/471,785 issued as U.S. Patent No. 10,036,012. No new matter is introduced by this amendment to the specification.

Applicant believes that the objection to the specification is overcome.

Objections to the Claims

The Office Action states on pages 3-4 that claims 51, 56-58, 61, and 64 are objected to because of informalities.

Applicant has herein amended claims 51, 56-58, 61, and 64 in accordance with the suggestions provided on pages 3-4 of the Office Action.

Applicant believes that the objections to the claims are overcome.

Rejections under 35 U.S.C. §112

The Office Action states on pages 4-5 that claims 59 and 66 is rejected under 35 U.S.C. §112, second paragraph as indefinite.

Applicant has herein amended claim 59 as suggested on page 5 of the Office Action. Accordingly, Applicant submits that claim 59 is definite and complies with the requirements of 35 U.S.C. §112, second paragraph.

Applicant has herein amended claim 66 to replace the term "appending" with ligating. Applicant concurrently cancelled claim 67. Accordingly, Applicant submits that claim 66 is definite and complies with the requirements of 35 U.S.C. §112, second paragraph.

Applicant requests that the rejections under 35 U.S.C. §112, second paragraph, be reconsidered and withdrawn.

Rejections under 35 U.S.C. §103

The Office Action states on page 6 that claims 51, 52, 54-61 and 63-70 are rejected under 35 U.S.C. §103 over US 2005/0142577 to Jones in view of Tucker, 2009, Am. J. Hum. Gen., 85: 142-154. The Office Action states on page 9 that claim 53 is rejected under 35 U.S.C. §103 over Jones in view of Tucker, and in further view of U.S. 7,741,463 to Gormley. The Office Action states on page 10 that claim 62 is rejected under 35 U.S.C. §103 over Jones in view of Tucker, 2011, Nature, 475: 348-352 by Rothberg.

Applicant submits that the claims are non-obvious over the cited references.

A determination of obviousness requires basic factual inquiries into the scope and content of the prior art as well as the difference between the claimed invention and the prior art. *Graham v. John Deere Co.*, 383 U.S. 1 (1996). For a claim to be obvious over a combination of references, there must be some articulated reason with a rational underpinning for why one would have made the proposed combination. *KSR Int'l Co. v. Teleflex, Inc.*, 550 U.S. 398, 418 (2007). The Office must consider objective evidence of non-obviousness, including evidence of unexpected results, skepticism of experts, and other 'secondary considerations'. *MPEP §716.01*. Such evidence may be presented in an affidavit or declaration, which is considered timely submitted when presented prior to a final Office Action. *MPEP §716.01; 37 C.F.R. §1.132(A)(1)*.

The claims recite sequencing methods in which a sequence of interest is enriched using a single target-specific oligonucleotide. The sequence of interest has a first adapter attached to one end. The target-specific oligonucleotide has a portion complementary to the sequence of interest and a non-complementary portion at the 5' end. This non-complementary portion contains a second adapter sequence. The oligonucleotide anneals to the target of interest and is extended to produce an extension product that has the sequence of interest flanked by the first and second adapters.

Each adapter contains a priming site. Thus, the enriched sequence of interest can be amplified using a common primer pair. The ability to use a common primer pair circumvents the need to use multiplex PCR with multiple primer pairs. *As-filed application, [0019]*.

Unexpectedly, the claimed methods result in low amounts of off-target sequence enrichment, a requirement for effective, massively parallel sequencing.

The claims are not obvious over the cited references because the combination of references do not teach enrichment with a single target-specific oligonucleotide and subsequent amplification using a common set of primers. Further, the references cannot be combined to arrive at Applicant's invention with a reasonable expectation of success.

Jones fails to disclose enrichment by extending a single target-specific oligonucleotide as claimed. In fact, Jones provides no disclosure of enrichment through an extension reaction. Jones likewise does not disclose the recited target-specific oligonucleotide with a non-complementary 5' adapter. Furthermore, Jones does not disclose sequencing, nor suggest that its teachings can extend to massively parallel sequencing.

Jones reports methods for preparing nucleic acids for microarray analysis. *Jones, [0002]*. Amplified products are hybridized to a microarray for analysis. *Id.* The methods use "capture probes" to incorporate amplification priming sites into target sequences. *Id., [0079] and [0087]*. These capture probes contain a "common sequence" that is complementary to a portion of a target sequence. *Id., [0005]*. Extension products with this complementary common sequence "are amplified by PCR using primers to the common sequences". *Id., [0079]*.

Unlike the claimed methods, Jones' disclosure does not use extension of a target-specific oligonucleotide for enrichment. Rather, Jones requires additional steps for sequence enrichment prior to amplification. *Id., [0011], [0087], [0102]-[0104], and FIG. 3*. Enrichment in Jones requires either positively selecting extended capture probes or depleting non-extended products. *Id.*

Jones also fails to disclose the recited target-specific oligonucleotide, which has a non-complementary second adapter that serves as a priming site. Rather, Jones' capture probes employ a "common sequence", complementary to the target sequence. The common sequences serve as priming sites for "common sequence primers". *Id., [0102]*.

Further, because Jones does not rely on a non-complementary adapter to provide a priming site, Jones cannot avoid using multiple primer pairs when amplifying different target

sequences. Jones' priming sites (the "common sequence") are complementary to the target sequence. Thus, for each different target amplified, a different "common sequence" and "common sequence primer" is required.

Furthermore, Applicant respectfully notes that because Jones does not disclose sequencing, the disclosure is not relevant to the claimed methods.

A person of skill in the art would not find Jones' disclosure applicable to sequencing because sequencing demands less off-target enrichment than microarray hybridization. Consequently, one would not have a reasonable expectation of success in applying Jones' enrichment methods to massively parallel sequencing.

Microarray hybridization is far more tolerant of off-target sequence enrichment than is massively-parallel sequencing. This is due to the fundamental nature of microarray hybridization assays, in which many amplified products are hybridized to spots on an array and their aggregate signals detected. Because microarrays rely on hybridization to probes, off-target amplicons may simply not find a complementary probe to bind to on the array. This precludes the off-target products from impacting the analysis. Similarly, because the aggregate signal of many products are detected, signals from any off-target products on the array fall into the background compared to the intense aggregate signals produced by the on-target products hybridized to the array.

In contrast, in massively parallel sequencing, off-target amplicons are sequenced just like on-target amplicons. As a result, off-target amplicons can flood the sequencing system, confounding sequence analysis. Sequencing lacks the intrinsic abilities of microarray hybridization to overcome off-target enrichment, and nothing in Jones suggests otherwise.

Accordingly, because Jones does not disclose sequencing, but rather microarray-based techniques, Jones' enrichment techniques could not be applied to sequencing with a reasonable expectation of success.

This conclusion is supported in the attached expert declaration of Dr. Brooks, filed in the parent of the subject application, U.S. 15/471,785. *Brooks Declaration*, ¶¶14-17.

Gromley does not cure Jones' omissions.

Gormley provides methods for generating nucleic acid libraries by *ligating two adapters* to a target sequence. *Gromley*, Col. 6, lines 48-59. The adapters contain a priming site, and "a key feature of the method of the invention [is] that the initial primer extension reaction is carried out using a 'universal primer' which binds specifically to a cognate sequence within an adapter

portion of the adapter-target construct, and is not carried out using a target-specific primer". *Gormley, col. 12, lines 22-27*. Thus, Gormley actually teaches away from the claimed methods, which use a target-specific oligonucleotide for the initial extension and incorporating a second adapter.

Tucker is merely an overview of sequencing techniques. *Tucker, page 142*. Tucker provides an overview of enrichment techniques used in sequencing. *Id., Figure 6*. However, not a single one of these methods enriches a target sequence using a single target-specific oligonucleotide.

Rothberg reports semiconductor sequencing. *Rothberg, abstract*. Rothberg only teaches a method of enrichment in which "unique forward and reverse adapters were ligated" to target sequences. *Rothberg Supplement, p. 22*.

Thus, the combination of references fails to teach all claimed elements as required to reject the claims as obvious.

Further, Applicant notes that an expert in the relevant art expressed disbelief that the claimed sequencing methods would provide adequate specificity for use with massively parallel sequencing. As the MPEP states, "[e]xpressions of disbelief by experts constitute strong evidence of nonobviousness". *MPEP §716.05* (quoting *Environmental Designs, Ltd. v. Union Oil Co. of Cal.*, 713, F.2d 693, 698 (Fed. Cir. 1983)).

In the attached declaration, Dr. Brooks explains that in 2012 he was approached to use the claimed methods. *Brooks Declaration, ¶14*. At that time, Dr. Brooks believed the methods would provide poor target specificity, which would lead to poor quality sequencing data. *Id.* Dr. Brooks thought target-specific primers would bind to off-target sites at a relatively high rate, leading to non-specific enriched nucleic acid sequences that would flood the sequencing system and confound sequence analysis. *Id.* Dr. Brooks felt that this off-target activity would affect the ability to create a uniform distribution of on-target sequences, which would lead, not only to an incomplete or less complex library, but one that had such an uneven distribution of targets that it would severely impair quantitative analyses. *Id., ¶15*.

Importantly, none of the cited references provide a disclosure that contradicts Dr. Brook's initial skepticism that the claimed methods would work.

Applicant further notes that the claimed methods provided unexpected results, and "[e]vidence of unobvious or unexpected advantages or properties, can rebut *prima facie* obviousness". *MPEP* §716.02(a).

In the attached declaration, Dr. Brooks provides evidence that the claimed methods provided unexpected and advantageous results.

Owing to his initial skepticism, Dr. Brooks expected to observe about fifty percent of sequence reads having the sequence of interest when using the claimed methods. *Brooks Declaration*, ¶16. However, unexpectedly, when using the methods, Dr. Brooks observed that over ninety percent of the sequence reads contained on-target sequences. *Id.* Dr. Brooks explained that this finding was unexpected, and that he would never have thought to use the claimed methods unless specifically asked to do so. *Id.*

Further, nothing in the cited references would render this as an expected result.

As discussed above, Dr. Brooks stated that Jones' microarray-based disclosure was inapplicable to massively-parallel sequencing. *Id.*, ¶17. Thus, nothing in Jones would render the success of the claimed methods as an expected result. Furthermore, Jones required separate enrichment steps.

Not only are the claimed results unexpected over Gormley, as explained, Gormley actually teaches away from the claimed methods.

Tucker or Rothberg never even mention enrichment using a target-specific oligonucleotide as claimed, and therefore do not render the results expected.

Based on the foregoing remarks and the attached declaratory evidence, Applicant submits that the claims are non-obvious. Accordingly, Applicant respectfully requests that the rejections under 35 U.S.C. §103 be reconsidered and withdrawn.

Double Patenting

The Office Action states on page 12 that claims 51-62 and 64-69 are rejected on the ground of non-statutory double patenting over claims 1-37 of U.S. Patent No. 9,650,628. The Office Action states on page 14 that claims 63 and 70 are rejected on the ground of non-statutory double patenting over claims 1-37 of U.S. Patent No. 9,650,628 in view of Tucker (The American Journal of Human Genetics 2009; 85: 142-154). The Office Action states on page 15 that claims 51-62 and 64-69 are rejected on the ground of non-statutory double patenting over

claims 1-22 of U.S. Patent No. 10,036,012. The Office Action states on page 17 that claims 63 and 70 are rejected on the ground of non-statutory double patenting over claims 1-22 of U.S. Patent No. 10,036,012 in view of Tucker.

Applicant is filing herewith a terminal disclaimer in compliance with 37 C.F.R. §1.321 over U.S. Patent Nos. 9,650,628 and 10,036,012. Accordingly, Applicant submits that the rejections on the ground of non-statutory double patenting are overcome.

Conclusion

Applicant submits that the pending claims are in a condition for allowance, and Applicant requests a Notice of Allowance.

Dated: July 28, 2020

Respectfully submitted,
BROWN RUDNICK LLP

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63824294 v1

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Electronic Acknowledgement Receipt

EFS ID:	40121626
Application Number:	16017340
International Application Number:	
Confirmation Number:	2635
Title of Invention:	COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION
First Named Inventor/Applicant Name:	Doug AMORESE
Customer Number:	21710
Filer:	Thomas C. Meyers/Jeanne Waters
Filer Authorized By:	Thomas C. Meyers
Attorney Docket Number:	NUGN-037/04US 34079/440
Receipt Date:	28-JUL-2020
Filing Date:	25-JUN-2018
Time Stamp:	15:43:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment		no			
File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		NUGN-037-04US_Amendment_4-30-20_NFOA.pdf	204834	yes	12
			11e10160822b27ccd091e0af6d30113fb4c81616		

	Multipart Description/PDF files in .zip description		
	Document Description	Start	End
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Specification	2	2
	Claims	3	5
	Amendment/Req. Reconsideration-After Non-Final Reject	6	12

Warnings:

Information:

2	Affidavit-traversing rejectns or objectns rule 132	NUGN-037-04US_Declaration. PDF	1337403	no	23
			d1c8c8082c9672ecb934aefa4760f460e88c5943		

Warnings:

Information:

Total Files Size (in bytes):		1542237
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: NUGEN TECHNOLOGIES, INC. ART UNIT: 1637
SERIAL NUMBER: 16/017,340 CONF. NO.: 2635
FILING DATE: June 25, 2018 EXAMINER: Angela Marie Bertagna

TITLE: **COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION**

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

FILED ELECTRONICALLY

AMENDMENT AND RESPONSE

This Response is in reply to the non-final Office Action mailed April 30, 2020, from the United States Patent and Trademark Office. Applicant is filing a terminal disclaimer herewith and authorizes the require fee to be charged to Deposit Account No. 50-0369. Applicant believes that no other fee is due with this response. Nevertheless, Applicant authorizes any required fee to be charged to Deposit Account No. 50-0369.

Amendments to the specification begin on page 2.

Amendments to the claims begin on page 3.

Remarks begin on page 6.

AMENDMENTS TO THE SPECIFICATION

Please amend the as-filed specification at page 1, paragraph [0001], as follows:

This application is a continuation of U.S. application Ser. No. 15/471,785, filed Mar. 28, 2017, now U.S. Pat. No. 10,036,012, which claims the benefit of U.S. application Ser. No. 13/750,768, filed Jan. 25, 2013, now U.S. Pat. No. 9,650,628, which claims the benefit of U.S. Provisional Application No. 61/591,241, filed Jan. 26, 2012, all of which are incorporated herein by reference in their entirety.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875				Application or Docket Number 16/017,340		Filing Date 06/25/2018		☐ To be Mailed				
ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO												
APPLICATION AS FILED - PART I												
		(Column 1)	(Column 2)									
FOR		NUMBER FILED	NUMBER EXTRA		RATE (\$)		FEE (\$)					
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))		N/A	N/A		N/A							
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))		N/A	N/A		N/A							
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))		N/A	N/A		N/A							
TOTAL CLAIMS (37 CFR 1.16(i))		minus 20 = *		x \$100 =								
INDEPENDENT CLAIMS (37 CFR 1.16(h))		minus 3 = *		x \$460 =								
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))		If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).										
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))												
* If the difference in column 1 is less than zero, enter "0" in column 2.						TOTAL						
APPLICATION AS AMENDED - PART II												
		(Column 1)	(Column 2)		(Column 3)							
AMENDMENT	07/28/2020	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)				
	Total (37 CFR 1.16(i))	* 19	Minus	** 20	= 0	x \$100 =		0				
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0	x \$460 =		0				
	☐ Application Size Fee (37 CFR 1.16(s))											
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))											
								TOTAL ADD'L FEE		0		
AMENDMENT												
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)				
	Total (37 CFR 1.16(i))	*	Minus	**	=	x \$0 =						
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x \$0 =						
	☐ Application Size Fee (37 CFR 1.16(s))											
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))											
								TOTAL ADD'L FEE				
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.						LIE						
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".						/CORALIA BETANCOURT/						
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".												
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.												

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: NUGEN TECHNOLOGIES, INC. ART UNIT: 1637
SERIAL NUMBER: 16/017,340 CONF. NO.: 2635
FILING DATE: June 25, 2018 EXAMINER: Angela Marie Bertagna

TITLE: **COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC
ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY
LIBRARY GENERATION**

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

FILED ELECTRONICALLY

TRANSMITTAL LETTER

This transmittal letter is accompanying an Electronic Terminal Disclaimer under 37 C.F.R. §1.321, filed herewith, over U.S. Patent Nos. 9,650,628 and 10,036,012. As explained below, no fee is due in connection with this Electronic Terminal Disclaimer.

On July 28, 2020, Applicant filed an electronic terminal disclaimer over U.S. Patent Nos. 9,650,628 and 10,036,012 via EFS-Web in connection with this application. Applicant also paid the requisite fee for the terminal disclaimer from Deposit Account No. 50-0369. An Electronic Acknowledgment Receipt never issued for filing the July 28, 2020 electronic terminal disclaimer.

Later, on July 28, 2020, Applicant's representative called the Patent Electronic Business Center at the U.S. Patent Office. The Office informed Applicant's representative that the July 28, 2020 electronic terminal disclaimer and required fee were received by the U.S. Patent Office, and that an Electronic Acknowledgment Receipt should issue within hours.

On July 29, 2020, Applicant's representative discovered that the Electronic Acknowledgment Receipt had still not issued.

On July 29, 2020 Applicant's representative telephoned the Patent Electronic Business Center and spoke with Agent 41. The ID for this call is 1-705356966. Agent 41 explained that Applicant should file a new electronic terminal disclaimer with a transmittal letter explaining that the fee for the terminal disclaimer had already been paid.

Accordingly, Applicant is filing herewith a new Electronic Terminal Disclaimer without the fee.

If the Examiner has any questions regarding this application, the Examiner is encouraged to contact Applicant's undersigned representative.

Dated: July 29, 2020

Respectfully submitted,
BROWN RUDNICK LLP

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63825097 v1

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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) NUGN-037/04US
<p>In re Application of: Douglas A. Amorese et al.</p> <p>Application No.: 16/017,340-Conf. #2635</p> <p>Filed: June 25, 2018</p> <p>For: COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION</p> <p>The applicant, <u>NUGEN TECHNOLOGIES, INC.</u>, owner of <u>100</u> percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent No. <u>(see attached)</u> as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.</p> <p>In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:</p> <ul style="list-style-type: none">expires for failure to pay a maintenance fee;is held unenforceable;is found invalid by a court of competent jurisdiction;is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;has all claims canceled by a reexamination certificate;is reissued; oris in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer. <p>Check either box 1 or 2 below, if appropriate.</p> <p>1. ☐ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.</p> <p>I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p> <p>2. ☒ The undersigned is an attorney or agent of record. Reg. No. <u>36,989</u></p> <div style="display: flex; justify-content: space-between; margin-top: 20px;"><div style="width: 60%; text-align: center;"><u>/Thomas C. Meyers/</u> Signature</div><div style="width: 35%; text-align: center;"><u>July 29, 2020</u> Date</div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Thomas C. Meyers</u> Typed or printed name</div><div style="width: 35%;"></div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Attorney for Applicant(s)</u> Title</div><div style="width: 35%; text-align: center;"><u>(617) 856-8200</u> Telephone Number</div></div> <p>☐ Terminal disclaimer fee under 37 CFR 1.20(d) included.</p> <p style="text-align: center;">WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.</p>	

<p style="text-align: center;">SUPPLEMENTAL SHEET FOR TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT</p>	<p>Docket Number (Optional) NUGN-037/04US</p>
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Prior patent Nos. applicable to this terminal disclaimer (referenced on first page):

9650628
10036012

63825174 v1

Electronic Acknowledgement Receipt

EFS ID:	40140481
Application Number:	16017340
International Application Number:	
Confirmation Number:	2635
Title of Invention:	COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION
First Named Inventor/Applicant Name:	Doug AMORESE
Customer Number:	21710
Filer:	Thomas C. Meyers/Jeanne Waters
Filer Authorized By:	Thomas C. Meyers
Attorney Docket Number:	NUGN-037/04US 34079/440
Receipt Date:	29-JUL-2020
Filing Date:	25-JUN-2018
Time Stamp:	16:26:12
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer Filed	NUGN-03-04US_TERMINAL-DISCLAIMER2.pdf	111313 0cca720fd0e1f3b5a5139ba34ef87c843a1d202a	no	2

Warnings:

Information:					
2	Miscellaneous Incoming Letter	NUGN-037-04US_TD_Transmittal_LE.pdf	117369	no	2
			75303e10c292ba13083e8118e388b6f2b72a8107		
Warnings:					
Information:					
Total Files Size (in bytes):				228682	
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					

Application Number * 16/017,340 *	Application/Control No. 16/017,340	Applicant(s)/Patent under Reexamination AMORESE et al.	
	Examiner BERTAGNA, ANGELA MARIE	Art Unit 1637	
Document Code - DISQ		Internal Document - DO NOT MAIL	

TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed: <u>29 July 2020</u>	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by:
/FELICIA D ROBERTS/ Technology Center: <u>OPLC</u> Telephone: <u>(571)272-0550</u> _____

PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L306	23	(Amorese near2 Doug\$).in. AND (ligat\$ OR ligase).clm. AND (adapt\$ OR linker).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	OFF	2020/08/12 04:18 PM
L307	16	(armour near2 chris\$).in. AND (ligat\$ OR ligase).clm. AND (adapt\$ OR linker).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	OFF	2020/08/12 04:19 PM
L308	9	L307 NOT L306	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	OFF	2020/08/12 04:20 PM
L309	11	(nurith near2 kurn).in. AND (ligat\$ OR ligase).clm. AND (adapt\$ OR linker).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	OFF	2020/08/12 04:20 PM
L310	3	L309 NOT L307 NOT L306	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	OFF	2020/08/12 04:20 PM
L311	17	(nugen).as. AND (ligat\$ OR ligase).clm. AND (adapt\$ OR linker).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	OFF	2020/08/12 04:20 PM
L312	7	L311 NOT L309 NOT L307 NOT L306	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	OFF	2020/08/12 04:20 PM
L313	431	((ligat\$) near8 (adaptor OR linker OR adapter)) SAME ((primer OR oligonucleotide OR oligo) near8 (specific) near8 (target OR template OR interest) near8 (tail OR tailed OR tag OR tagged OR adapter OR adaptor OR linker)) SAME ((pcr OR polymerase OR amplif\$) near8 (adaptor OR adapter OR linker) near8 primer)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:22 PM
L314	204	((ligat\$) near8 (adaptor OR linker OR adapter)) SAME ((primer OR oligonucleotide OR oligo) near8 (specific) near8 (target OR template OR interest) near8 (tail OR	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:22 PM

		tailed OR tag OR tagged OR adapter OR adaptor OR linker)) SAME ((pcr OR polymerase OR amplif\$) near8 (adaptor OR adapter OR linker) near8 primer) AND @ay<=2012					
L315	1319	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) near3 (target OR template OR gene) near3 specific near3 primer) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:23 PM
L316	95	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:23 PM
L317	183	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	ON	2020/08/12 04:23 PM
L318	43	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	ON	2020/08/12 04:24 PM
L319	68	((ligat\$) near3 (adapter OR adaptor OR linker)) SAME (selectiv\$ near3 (pcr OR polymerase OR amplif\$) near3 (target OR template OR genomic)) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:24 PM

L320	21	((ligat\$) near5 (adaptor OR linker OR adapter)) SAME ((primer OR oligonucleotide OR oligo) near5 (specific) near5 (target OR template OR interest) near5 (tail OR tailed OR tag OR tagged OR adapter OR adaptor OR linker)) SAME ((pcr OR polymerase OR amplif\$) near5 (adaptor OR adapter OR linker) near5 primer) SAME (sequencing OR sequenced) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:24 PM
L321	43	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:25 PM
L322	33	((ligat\$) near3 (adapter OR adaptor OR linker)) SAME ((primer) near3 (tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) near3 (target near3 specific\$)) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:26 PM
L323	43	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:26 PM
L324	29	(ligat\$ OR ligase OR attach\$ OR append\$) near8 (adaptor OR adapter OR linker OR linking) near8 ((extended	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:26 PM

		OR extension) near3 (product OR oligonucleotide)) SAME (amplif\$ OR pcr OR (polymerase adj1 chain)) AND (primer near5 (tag OR tagged OR tail OR tailed OR "not complementary" OR (non adj1 complementary))) AND @ay<=2012					
L325	94	((ligat\$) near5 (adaptor OR linker OR adapter)) SAME ((primer OR oligonucleotide OR oligo) near5 (specific) near5 (target OR template OR interest) near5 (tail OR tailed OR tag OR tagged OR adapter OR adaptor OR linker)) SAME ((pcr OR polymerase OR amplif\$) near5 (adaptor OR adapter OR linker) near5 primer) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:27 PM
L326	95	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:27 PM
L327	43	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:27 PM
L328	68	((ligat\$) near3 (adapter OR adaptor OR linker)) SAME (selectiv\$ near3 (pcr OR polymerase OR amplif\$) near3 (target OR template OR genomic)) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:28 PM
L329	21	((ligat\$) near5 (adaptor OR linker OR adapter))	US-PGPUB; USPAT; FPRS; EPO; JPO;	OR	ON	ON	2020/08/12 04:28 PM

		SAME ((primer OR oligonucleotide OR oligo) near5 (specific) near5 (target OR template OR interest) near5 (tail OR tailed OR tag OR tagged OR adapter OR adaptor OR linker)) SAME ((pcr OR polymerase OR amplif\$) near5 (adaptor OR adapter OR linker) near5 primer) SAME (sequencing OR sequenced) AND @ay<=2012	DERWENT				
L330	43	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:28 PM
L331	33	((ligat\$) near3 (adapter OR adaptor OR linker)) SAME ((primer) near3 (tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) near3 (target near3 specific\$)) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:28 PM
L332	43	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:28 PM
L333	29	(ligat\$ OR ligase OR attach\$ OR append\$) near8 (adaptor OR adapter OR linker OR linking) near8 ((extended OR extension) near3 (product OR	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	ON	2020/08/12 04:29 PM

		oligonucleotide)) SAME (amplif\$ OR pcr OR (polymerase adj1 chain)) AND (primer near5 (tag OR tagged OR tail OR tailed OR "not complementary" OR (non adj1 complementary))) AND @ay<=2012					
L334	1498	(C12N15/1068 OR C12N15/66 OR C12N15/1072 OR C12Q2525/191 OR C12Q1/6869 OR C40B30/04 OR C12Q2535/122 OR C12Q1/6844 OR C12Q1/6855 OR C40B50/06).cpc. AND ((ligat\$ near3 (adapt\$r OR linker)) AND (primer near3 (adapt\$r OR linker))) AND @ay<=2012	US-PGPUB; USPAT; USOCR; FIT (CN, FR, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	ON	2020/08/12 04:30 PM
L335	930	(C12N15/1068 OR C12N15/66 OR C12N15/1072 OR C12Q2525/191 OR C12Q1/6869 OR C40B30/04 OR C12Q2535/122 OR C12Q1/6844 OR C12Q1/6855 OR C40B50/06).cpc. AND ((ligat\$ near3 (adapt\$r OR linker)) SAME (primer near3 (adapt\$r OR linker))) AND @ay<=2012	US-PGPUB; USPAT; USOCR; FIT (CN, FR, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	ON	2020/08/12 04:31 PM
L336	867	(C12N15/1068 OR C12N15/66 OR C12N15/1072 OR C12Q2525/191 OR C12Q1/6869 OR C40B30/04 OR C12Q2535/122 OR C12Q1/6844 OR C12Q1/6855 OR C40B50/06).cpc. AND ((ligat\$ near3 (adapt\$r OR linker)) SAME (primer near3 (adapt\$r OR linker))) AND (sequencing OR sequenced) AND @ay<=2012	US-PGPUB; USPAT; USOCR; FIT (CN, FR, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	ON	2020/08/12 04:31 PM
L337	813	(C12N15/1068 OR	US-PGPUB; USPAT;	OR	ON	ON	2020/08/12

		C12N15/66 OR C12N15/1072 OR C12Q2525/191 OR C12Q1/6869 OR C40B30/04 OR C12Q2535/122 OR C12Q1/6844 OR C12Q1/6855 OR C40B50/06).cpc. AND (((ligation OR ligated OR ligate) near3 (adapt\$r OR linker)) SAME (primer near3 (adapt\$r OR linker))) AND (sequencing OR sequenced) AND @ay<=2012	USOCR; FIT (CN, FR, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB				04:31 PM
L338	0	(C12N15/66 SAME C12Q2525/191).cpcc. AND (((ligation OR ligated OR ligate) near3 (adapt\$r OR linker)) SAME (primer near3 (adapt\$r OR linker))) AND (sequencing OR sequenced) AND @ay<=2012	US-PGPUB; USPAT; USOCR; FIT (CN, FR, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	ON	2020/08/12 04:32 PM
L339	24	(C12N15/66 SAME C12Q2525/191).cpcc.	US-PGPUB; USPAT; USOCR; FIT (CN, FR, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	ON	2020/08/12 04:32 PM
L340	0	(C12N15/66 SAME C12Q2525/191).cpcc. AND @ay<=2012	US-PGPUB; USPAT; USOCR; FIT (CN, FR, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	ON	2020/08/12 04:34 PM
L341	1	(C12N15/1068 SAME C12Q2525/191 SAME C12Q2563/185).cpcc.	US-PGPUB; USPAT; USOCR; FIT (CN, FR, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	ON	2020/08/12 04:34 PM

PE2E SEARCH - Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
N262	23	(Amorese near2 Doug\$.in. AND (ligat\$ OR ligase).clm. AND (adapt\$ OR linker).clm.	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:37 PM
N263	0	(C12N15/1068 SAME C12Q2525/191 SAME C12Q2563/185).cpcc.	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:37 PM

N264	7	(C12N15/66 SAME C12Q2525/191).cpcc.	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:37 PM
N265	0	(C12N15/66 SAME C12Q2525/191).cpcc. AND (((ligation OR ligated OR ligate) near3 (adapt\$r OR linker)) SAME (primer near3 (adapt\$r OR linker))) AND (sequencing OR sequenced) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:38 PM
N266	28	(ligat\$ OR ligase OR attach\$ OR append\$) near8 (adaptor OR adapter OR linker OR linking) near8 ((extended OR extension) near3 (product OR oligonucleotide)) SAME (amplif\$ OR pcr OR (polymerase adj1 chain)) AND (primer near5 (tag OR tagged OR tail OR tailed OR "not complementary" OR (non adj1 complementary))) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:38 PM
N267	42	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:38 PM
N268	30	((ligat\$) near3 (adapter OR adaptor OR linker)) SAME ((primer) near3 (tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) near3 (target near3 specific\$)) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:38 PM
N269	42	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:38 PM

N270	20	primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:38 PM
N271	56	((ligat\$) near5 (adaptor OR linker OR adapter)) SAME ((primer OR oligonucleotide OR oligo) near5 (specific) near5 (target OR template OR interest) near5 (tail OR tailed OR tag OR tagged OR adapter OR adaptor OR linker)) SAME ((pcr OR polymerase OR amplif\$) near5 (adaptor OR adapter OR linker) near5 primer) SAME (sequencing OR sequenced) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:38 PM
N272	56	((ligat\$) near3 (adapter OR adaptor OR linker)) SAME (selectiv\$ near3 (pcr OR polymerase OR amplif\$) near3 (target OR template OR genomic)) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:39 PM
N273	42	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:39 PM
N274	93	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker)	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:39 PM

N275	88	NEAR (target OR template OR gene) NEAR specific NEAR primer) AND @ay<=2012 ((ligat\$) near5 (adaptor OR linker OR adapter)) SAME ((primer OR oligonucleotide OR oligo) near5 (specific) near5 (target OR template OR interest) near5 (tail OR tailed OR tag OR tagged OR adapter OR adaptor OR linker)) SAME ((pcr OR polymerase OR amplif\$) near5 (adaptor OR adapter OR linker) near5 primer) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:39 PM
N276	28	(ligat\$ OR ligase OR attach\$ OR append\$) near8 (adaptor OR adapter OR linker OR linking) near8 ((extended OR extension) near3 (product OR oligonucleotide)) SAME (amplif\$ OR pcr OR (polymerase adj1 chain)) AND (primer near5 (tag OR tagged OR tail OR tailed OR "not complementary" OR (non adj1 complementary))) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:40 PM
N277	42	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:40 PM
N278	30	((ligat\$) near3 (adapter OR adaptor OR linker)) SAME ((primer) near3 (tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) near3 (target near3 specific\$)) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:40 PM

N279	42	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:40 PM
N280	20	((ligat\$) near5 (adaptor OR linker OR adapter)) SAME ((primer OR oligonucleotide OR oligo) near5 (specific) near5 (target OR template OR interest) near5 (tail OR tailed OR tag OR tagged OR adapter OR adaptor OR linker)) SAME ((pcr OR polymerase OR amplif\$) near5 (adaptor OR adapter OR linker) near5 primer) SAME (sequencing OR sequenced) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:40 PM
N281	56	((ligat\$) near3 (adapter OR adaptor OR linker)) SAME (selectiv\$ near3 (pcr OR polymerase OR amplif\$) near3 (target OR template OR genomic)) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:40 PM
N282	42	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:41 PM
N283	180	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:41 PM

		primer) SAME (amplif\$ OR polymerase OR pcr) SAME ((adaptor OR adapter OR linker OR universal OR common) near3 primer)					
N284	93	((tag OR tagged OR tail OR tailed OR adapter OR adaptor OR linker) NEAR (target OR template OR gene) NEAR specific NEAR primer) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:41 PM
N285	187	((ligat\$) near8 (adaptor OR linker OR adapter)) SAME ((primer OR oligonucleotide OR oligo) near8 (specific) near8 (target OR template OR interest) near8 (tail OR tailed OR tag OR tagged OR adapter OR adaptor OR linker)) SAME ((pcr OR polymerase OR amplif\$) near8 (adaptor OR adapter OR linker) near8 primer) AND @ay<=2012	US-PGPUB; USPAT	OR	ON	ON	2020/08/12 04:41 PM
N286	11	(nugen).as. AND (ligat\$ OR ligase).clm. AND (adapt\$ OR linker).clm.	US-PGPUB; USPAT	OR	OFF	OFF	2020/08/12 04:41 PM
N287	11	(nurith near2 kurn).in. AND (ligat\$ OR ligase).clm. AND (adapt\$ OR linker).clm.	US-PGPUB; USPAT	OR	OFF	OFF	2020/08/12 04:41 PM
N288	16	(armour near2 chris\$).in. AND (ligat\$ OR ligase).clm. AND (adapt\$ OR linker).clm.	US-PGPUB; USPAT	OR	OFF	OFF	2020/08/12 04:42 PM
N289	23	(Amorese near2 Doug\$).in. AND (ligat\$ OR ligase).clm. AND (adapt\$ OR linker).clm.	US-PGPUB; USPAT	OR	OFF	OFF	2020/08/12 04:42 PM



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NOTICE OF ALLOWANCE AND FEE(S) DUE

21710 7590 08/19/2020
BROWN RUDNICK LLP
ONE FINANCIAL CENTER
BOSTON, MA 02111

EXAMINER	
BERTAGNA, ANGELA MARIE	
ART UNIT	PAPER NUMBER

1637

DATE MAILED: 08/19/2020

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/017,340	06/25/2018	Doug AMORESE	NUGN-037/04US 34079/440	2635

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$0.00	\$1000	11/19/2020

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

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Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

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(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/017,340	06/25/2018	Doug AMORESE	NUGN-037/04US 34079/440	2635

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$0.00	\$1000	11/19/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
BERTAGNA, ANGELA MARIE	1637	435-006100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

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- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☐ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

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5. Change in Entity Status (from status indicated above)

- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/017,340	06/25/2018	Doug AMORESE	NUGN-037/04US 34079/440	2635
21710	7590	08/19/2020	EXAMINER	
BROWN RUDNICK LLP ONE FINANCIAL CENTER BOSTON, MA 02111			BERTAGNA, ANGELA MARIE	
			ART UNIT	PAPER NUMBER
			1637	
DATE MAILED: 08/19/2020				

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b) (2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 16/017,340		Applicant(s) AMORESE et al.	
	Examiner Angela Bertagna		Art Unit 1637	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to Applicant's responses filed on 7/28/20 and 7/29/20.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are 51-66 and 68-70. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☐ All b) ☐ Some *c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. ____.

3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: ____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date ____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)	5. ☒ Examiner's Amendment/Comment
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date ____.	6. ☐ Examiner's Statement of Reasons for Allowance
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material ____.	7. ☐ Other ____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. ____.	

/ANGELA M. BERTAGNA/ Primary Examiner, Art Unit 1637	
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DETAILED CORRESPONDENCE

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Status of the Application

2. Applicant's response filed on July 28, 2020 has been entered. Claims 51-66 and 68-70 are pending.

As discussed in greater detail below, Applicant's response has overcome all of the outstanding issues.

Terminal Disclaimer

3. The terminal disclaimer filed on July 29, 2020 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of US 10,036,012 B2 has been reviewed and is accepted. The terminal disclaimer has been recorded.

The terminal disclaimer filed on July 29, 2020 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of US 9,650,628 B2 has been reviewed and is accepted. The terminal disclaimer has been recorded.

Response to Amendment

4. The declaration of Dr. Andrew Brooks, filed on July 28, 2020, under 37 CFR 1.132 is sufficient to overcome all of the rejections made previously under 35 U.S.C. 103(a) citing Jones and Tucker as the primary combination of references.

Response to Arguments

5. Applicant's arguments filed on July 28, 2020 have been fully considered.

Objection to the Specification

Applicant argues that the objection should be withdrawn in view of the amendment to the specification (Remarks, page 6).

This argument was persuasive. The objection has been withdrawn.

Claim Objections

Applicant argues that the objections to claims 51, 56-58, 61, and 64 should be withdrawn in view of the claim amendments (Remarks, page 6).

This argument was persuasive. All of the previously made objections have been withdrawn.

Rejection of claims 59 and 66 under pre-AIA 35 U.S.C. 112, second paragraph

Applicant argues that the rejection should be withdrawn in view of the claim amendments (Remarks, pages 6-7).

This argument was persuasive. The rejection has been withdrawn.

Rejections of claims 51-70 under pre-AIA 35 U.S.C. 103(a) citing Jones and Tucker as the primary combination of references

Applicant's argument, see pages 10-11 of the Remarks, that the rejections should be withdrawn in view of the declaration evidence, was persuasive. All of the obviousness rejections made previously have been withdrawn.

Applicant's additional arguments on pages 7-10 of the Remarks have been considered, but they are moot since the rejections have been withdrawn in view of the declaration evidence.

Double Patenting

Applicant argues that the nonstatutory double patenting rejections citing US 10,036,012 and US 9,650,628 should be withdrawn in view of the terminal disclaimer submission (Remarks, pages 11-12).

As noted above, the terminal disclaimer was proper. The rejections have been withdrawn accordingly.

Allowable Subject Matter

6. Claims 51-66 and 68-70 are allowed.

Conclusion

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Angela Bertagna whose telephone number is (571)272-8291.

The examiner can normally be reached on 8-5, M-F.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at <http://www.uspto.gov/interviewpractice>.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished

applications is available through Private PAIR only. For more information about the PAIR system, see <https://ppair-my.uspto.gov/pair/PrivatePair>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/ANGELA M. BERTAGNA/
Primary Examiner, Art Unit 1637

Issue Classification 	Application/Control No. 16/017,340	Applicant(s)/Patent Under Reexamination AMORESE et al.
	Examiner Angela Bertagna	Art Unit 1637

CPC						
Symbol					Type	Version
C12N	/	15	/	1068	F	2013-01-01
C12N	/	15	/	66	I	2013-01-01
C12N	/	15	/	1072	I	2013-01-01
C12Q	/	2525	/	191	A	2013-01-01

CPC Combination Sets								
Symbol					Type	Set	Ranking	Version
C12N	/	15	/	66	I	1	1	2013-01-01
C12Q	/	2525	/	191	I	1	2	2013-01-01

NONE (Assistant Examiner)		Total Claims Allowed: 19	
/ANGELA M. BERTAGNA/ Primary Examiner, Art Unit 1637 (Primary Examiner)		12 August 2020 (Date)	
		O.G. Print Claim(s) 1	O.G. Print Figure NONE

Issue Classification 	Application/Control No. 16/017,340	Applicant(s)/Patent Under Reexamination AMORESE et al.
	Examiner Angela Bertagna	Art Unit 1637

INTERNATIONAL CLASSIFICATION			
CLAIMED			
C12N15/10	/	15	/ 10
C12N15/66	/	15	/ 66
NON-CLAIMED			
	/		/

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	19	
/ANGELA M. BERTAGNA/ Primary Examiner, Art Unit 1637	12 August 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

Issue Classification 	Application/Control No. 16/017,340	Applicant(s)/Patent Under Reexamination AMORESE et al.
	Examiner Angela Bertagna	Art Unit 1637

☐ Claims renumbered in the same order as presented by applicant
 ☐ CPA
 ☒ T.D.
 ☐ R.1.47

CLAIMS															
Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		10		19		28		37		46	5	55	14	64
	2		11		20		29		38		47	6	56	15	65
	3		12		21		30		39		48	7	57	16	66
	4		13		22		31		40		49	8	58		67
	5		14		23		32		41		50	9	59	17	68
	6		15		24		33		42	1	51	10	60	18	69
	7		16		25		34		43	2	52	11	61	19	70
	8		17		26		35		44	3	53	12	62		
	9		18		27		36		45	4	54	13	63		

NONE (Assistant Examiner) (Date)		Total Claims Allowed: 19	
/ANGELA M. BERTAGNA/ Primary Examiner, Art Unit 1637 (Primary Examiner) (Date)		12 August 2020 (Date)	O.G. Print Claim(s) 1
			O.G. Print Figure NONE

<i>Search Notes</i> 	Application/Control No. 16/017,340	Applicant(s)/Patent Under Reexamination AMORESE et al.
	Examiner Angela Bertagna	Art Unit 1637

CPC - Searched*		
Symbol	Date	Examiner
C12N 15/1068	8/12/20	amb
C12N 15/66	8/12/20	amb
C12N 15/1072	8/12/20	amb
C12Q 2525/191	8/12/20	amb
C40B 30/04	8/12/20	amb
C12Q 2535/122	8/12/20	amb
C12Q 1/6844	8/12/20	amb
C12Q 1/6855	8/12/20	amb
C40B 50/06	8/12/20	amb

CPC Combination Sets - Searched*		
Symbol	Date	Examiner
C12N 15/66; C12Q 2525/191	8/12/20	amb
C12N 15/1068; C12Q 2525/191; C12Q 2563/185	8/12/20	amb

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

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<i>Search Notes</i> 	Application/Control No. 16/017,340	Applicant(s)/Patent Under Reexamination AMORESE et al.
	Examiner Angela Bertagna	Art Unit 1637

Search Notes		
Search Notes	Date	Examiner
searched all inventors by name in DAV	04/25/2020	amb
EAST search history attached (text & inventor name)	04/25/2020	amb
reviewed parent cases	04/25/2020	amb
updated inventor name search in DAV	08/12/2020	amb
updated search in PE2E-Search (text, inventor name, assignee)	08/12/2020	amb
All symbols listed in the "CPC-searched" section were combined with text (see PE2E-Search history printout)	8/12/20	amb
Symbols in each "combination sets" row were searched together (see PE2E Search history printout)	8/12/20	amb

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<i>Search Notes</i> 	Application/Control No. 16/017,340	Applicant(s)/Patent Under Reexamination AMORESE et al.
	Examiner Angela Bertagna	Art Unit 1637

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
searched USPAT & USPGPUBS (text, assignee, & inventor name)		08/12/2020	amb
searched all symbols listed in the "CPC-searched" section above in combination with text (see search history)		8/12/20	amb
searched the two combination sets listed above in the "combination sets" section (see search history)		8/12/20	amb

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Bibliographic Data

Application No: 16/017,340

Foreign Priority claimed: ☐ Yes ☒ No

35 USC 119 (a-d) conditions met: ☐ Yes ☒ No ☐ Met After Allowance

Verified and Acknowledged: /ANGELA M. BERTAGNA/

Examiner's Signature

Initials

Title:

COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC
ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY
LIBRARY GENERATION

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
06/25/2018	435	1637	NUGN-037/04US 34079/440
RULE			

APPLICANTS

NuGEN Technologies, Inc., San Carlos, CA, UNITED STATES

INVENTORS

Doug AMORESE, Los Altos, CA, UNITED STATES

Chris ARMOUR, Kirkland, WA, UNITED STATES

Nurith KURN, Palo Alto, CA, UNITED STATES

CONTINUING DATA

This application is a CON of 15471785 03/28/2017 PAT 10036012

15471785 is a CON of 13750768 01/25/2013 PAT 9650628

13750768 has PRO of 61591241 01/26/2012

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

07/12/2018

STATE OR COUNTRY

UNITED STATES

ADDRESS

BROWN RUDNICK LLP
ONE FINANCIAL CENTER
BOSTON, MA 02111
UNITED STATES

FILING FEE RECEIVED

\$3,280

PART B-FEE(S) TRANSMITTAL

Complete and send this form, together with the applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

By fax, send to: (571) 273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

21710 7590 08/19/2020
Brown Rudnick LLP
One Financial Center
Boston, Massachusetts 02111

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

Sandra Richards	(Typed or printed name)
/Sandra Richards/	(Signature)
November 18/2020	(Date)

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
16/017,340	06/25/2018	Douglas A. Amorese	NUGN-037/04US 34079/440	2635

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION

APPLN: TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1,000.00			\$1,000.00	11/19/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
A. M. Bertagna	1637	435-006.100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363) ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached. ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. Use of a Customer Number is required.	2. For printing on the patent front page, list (1) The names of up to 3 registered patent attorneys or agents OR, alternatively, (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.	1. Brown Rudnick LLP 2. Thomas C. Meyers 3.
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3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)
PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.
(A) NAME OF ASSIGNEE NUGEN TECHNOLOGIES, INC.
(B) RESIDENCE: (CITY and STATE or COUNTRY) Redwood City, California

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees Submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment (Please first reapply any previously paid fee shown above):

☒ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 50-0369

5. Change of Entity Status (from status indicated above) ☐ Applicant certifying micro entity status. See 37 CFR 1.29. ☐ Applicant asserting small entity status. See 37 CFR 1.27. ☐ Applicant changing to regular undiscounted fee status.	NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment. NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status. NOTE: Checking this box will be taken as a notification of loss of entitlement to small or micro entity status, as applicable.
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NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Thomas C. Meyers/ Date November 18, 2020
Typed or printed name Thomas C. Meyers Registration No. 36,989

Electronic Patent Application Fee Transmittal				
Application Number:		16017340		
Filing Date:		25-Jun-2018		
Title of Invention:		COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION		
First Named Inventor/Applicant Name:		Doug AMORESE		
Filer:		Thomas C. Meyers/Sandra Richards		
Attorney Docket Number:		NUGN-037/04US 34079/440		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1000	1000

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				1000

Electronic Acknowledgement Receipt

EFS ID:	41153480
Application Number:	16017340
International Application Number:	
Confirmation Number:	2635
Title of Invention:	COMPOSITIONS AND METHODS FOR TARGETED NUCLEIC ACID SEQUENCE ENRICHMENT AND HIGH EFFICIENCY LIBRARY GENERATION
First Named Inventor/Applicant Name:	Doug AMORESE
Customer Number:	21710
Filer:	Thomas C. Meyers/Sandra Richards
Filer Authorized By:	Thomas C. Meyers
Attorney Docket Number:	NUGN-037/04US 34079/440
Receipt Date:	18-NOV-2020
Filing Date:	25-JUN-2018
Time Stamp:	10:44:52
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 1000
RAM confirmation Number	E2020AHA45181328
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	NUGN-037-04US-Issue-Fee-Transmittal.pdf	35809	no	1
			aac8426f6941cb09a8bce9012add6ad576297518		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30593	no	2
			34f42cb89ec90537d3678efaa2bf9e34f740e6af6		
Warnings:					
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Total Files Size (in bytes):			66402		
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					



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ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 203 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

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