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- (71) Applicant: THE JOHNS HOPKINS UNIVERSITY
[US/US]; 3400 N. Charles Street, Baltimore, Maryland 21218 (US).
- (72) Inventors: GUERRERO-PRESTON, Rafael; 3000 Manhattan Avenue, Baltimore, Maryland 21215 (US).
SIDRANSKY, David; 7800 Seven Mile Lane, Baltimore, Maryland 21208 (US).
- (74) Agent: CONTRERA, Joseph; 100 N. Charles Street, 5th Floor, Baltimore, Maryland 21201 (US).

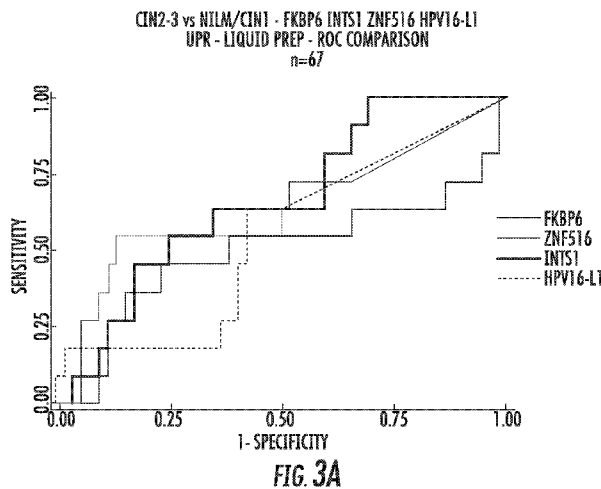
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(54) Title: METHOD TO USE VIRAL AND HOST METHYLATION MARKERS FOR CERVICAL CANCER SCREENING AND TRIAGE IN LIQUID PREP, SERUM/PLASMA, AND URINE: PCR AND SEQUENCING BASED PROCESS METHODS



(57) Abstract: Methods and kits for triaging a human papillomavirus (HPV)-positive woman into colposcopy are disclosed. The methods comprise determining the promoter methylation level of the promoter regions of a group of genes (e.g., viral and host genes) that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM).

METHOD TO USE VIRAL AND HOST METHYLATION MARKERS FOR
CERVICAL CANCER SCREENING AND TRIAGE IN LIQUID PREP,
SERUM/PLASMA, AND URINE: PCR AND SEQUENCING BASED PROCESS
METHODS

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REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 62/197,306, filed on July 27, 2015, which is hereby incorporated by reference for all purposes as if fully set forth herein.

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FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under grant numbers CA084986, and CA164092, awarded by the National Institutes of Health. The government has certain rights in the invention.

15

BACKGROUND

Cervical cancer screening is undergoing a major transformation with the adoption of testing for the presence of oncogenic human papilloma virus (HPV) types (1, 2). Persistent HPV infection of the cervical epithelium is a rare event because most infections are usually cleared without treatment (3). Yet a small percentage of HPV infections are associated to progression from low-grade squamous intraepithelial lesions (LSIL) to cervical intraepithelial neoplasia grade 3 (CIN3) lesions (4). In fact, persistent infection with at least one of the 13 carcinogenic types from the *alphapapillomavirus* genus (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) has been linked to cervical cancer(5). Close to 30% of CIN3 lesions progress to cervical cancer, but there are no agreed upon clinical tests of progression to cervical cancer (6, 7).

The new cervical cancer screening guidelines in the United States recommend HPV co-testing with Pap among women 30 years and older (8). However, clinical management for HPV-positive/Pap-negative women is not firmly established (9, 10). HPV has recently been shown to be a better indicator of cervical cancer risk than the Pap test (11). But even when co-testing with Pap and HPV has higher sensitivity and specificity than each separate test, together they cannot predict who will progress to cervical carcinoma (12, 13).

This led to the search of molecular markers of risk to progression to cervical cancer. HPV DNA methylation, like other DNA viruses, is correlated to progression to cancer (14). This seminal finding led to subsequent studies suggesting that detection of methylated HPV DNA may distinguish women with cervical
5 intraepithelial neoplasia grade 2-3 (CIN2+) from women with an oncogenic HPV type infection who show no evidence of CIN2+(15-19). In parallel, other groups examined the association between host DNA methylation and cervical cancer (20-23). Recent studies, have reported a positive association between CIN2+ and methylation of CpG sites in host and viral DNA isolated from liquid cytology samples (19, 20, 24).

10 Recently published reports have also explored the use of urine-based high risk HPV testing, as an alternative approach to liquid cytology for cervical cancer screening, in an attempt to identify less invasive cervical cancer screening technologies (25-27). Most of the studies have failed to attain clinical usefulness as they are limited by poor sensitivity, inappropriate protocols for DNA extraction from
15 circulating DNA in urine, small sample size, and the limit of detection of the HPV assays utilized (28-30). Most of the studies also fail to recognize the importance of using DNA isolation methods optimized to enrich for fragmented circulating cell-free DNA (ccfDNA) that crosses the kidneys and can be obtained in urine (31).

There is growing evidence that circulating short human, viral, and bacterial
20 DNA fragments from dying cells throughout the body, approximately 150-250 bases long on average, pass through the renal barrier and appear in urine as Transrenal DNA (TrDNA)(32) or ccfDNA. ccfDNA has been proposed as a tool for non-invasive prenatal monitoring, infectious disease monitoring, and tumor response monitoring (33-35). Recently, a capillary electrophoresis ccfDNA test that targets the *E1* region
25 of the HPV genome for the detection of high risk HPV demonstrated high sensitivity and modest specificity for urine-based detection of cervical pre-cancerous lesions(36).

Urine samples tested by the ccfDNA HPV capillary electrophoresis test had high concordance with corresponding cervical and urine samples tested by the widely used Linear Array HPV Genotyping Test (LA-HPV)(37). However, the ccfDNA
30 capillary electrophoresis HPV test is not quantitative, can only detect the presence or absence of high risk HPV and, similar to previously published urine based HPV tests, has limited specificity. None of the high-risk HPV urine-based reports use sequencing based approaches to quantify multiple HPV types, nor include methylated markers in their workflow to improve the sensitivity and specificity of the ccfDNA test.

SUMMARY

The practice of the present invention will typically employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant nucleic acid (e.g., DNA) technology, immunology, and RNA interference (RNAi) which are within the skill of the art. Non-limiting descriptions of certain of these techniques are found in the following publications: Ausubel, F., et al., (eds.), *Current Protocols in Molecular Biology*, *Current Protocols in Immunology*, *Current Protocols in Protein Science*, and *Current Protocols in Cell Biology*, all John Wiley & Sons, N.Y., edition as of December 2008; Sambrook, Russell, and Sambrook, *Molecular Cloning. A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001; Harlow, E. and Lane, D., *Antibodies—A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1988; Freshney, R. I., “Culture of Animal Cells, A Manual of Basic Technique”, 5th ed., John Wiley & Sons, Hoboken, N.J., 2005. Non-limiting information regarding therapeutic agents and human diseases is found in Goodman and Gilman's *The Pharmacological Basis of Therapeutics*, 11th Ed., McGraw Hill, 2005, Katzung, B. (ed.) *Basic and Clinical Pharmacology*, McGraw-Hill/Appleton & Lange 10th ed. (2006) or 11th edition (July 2009). Non-limiting information regarding genes and genetic disorders is found in McKusick, V. A.: *Mendelian Inheritance in Man. A Catalog of Human Genes and Genetic Disorders*. Baltimore: Johns Hopkins University Press, 1998 (12th edition) or the more recent online database: Online Mendelian Inheritance in Man, OMIM™. McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), as of May 1, 2010, World Wide Web URL: <http://www.ncbi.nlm.nih.gov/omim/> and in Online Mendelian Inheritance in Animals (OMIA), a database of genes, inherited disorders and traits in animal species (other than human and mouse), at <http://omia.angis.org.au/contact.shtml>.

In an aspect, the presently disclosed subject matter provides a method for triaging a human papillomavirus (HPV)-positive woman into colposcopy, the method comprising: (a) selecting a HPV-positive woman testing positive for one or more high risk types of HPV; (b) obtaining nucleic acid from a test sample from the selected HPV-positive woman; (c) determining in the nucleic acid from the test sample of the

selected HPV-positive woman a promoter methylation level of the promoter regions of a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM); and (d) triaging the HPV-positive woman into colposcopy when the level of promoter methylation of the group of genes is increased relative to the level of promoter methylation of the group of genes in a reference sample obtained from women having NILM.

In an aspect, the presently disclosed subject matter provides a method for triaging a human papillomavirus (HPV)-positive woman into colposcopy, the method comprising: (a) selecting a HPV-positive woman testing positive for one or more high risk types of HPV; (b) providing a kit that comprises the probes and/or primers needed for determining in the nucleic acid from a test sample of the selected HPV-positive woman a promoter methylation level of the promoter regions of a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM); (c) obtaining nucleic acid from the test sample from the selected HPV-positive woman; (d) determining the promoter methylation level in the nucleic acid from the test sample of the selected HPV-positive woman using the provided kit; and (e) triaging the HPV-positive woman into colposcopy when the level of promoter methylation of the group of genes is increased relative to the level of promoter methylation of the group of genes in a reference sample obtained from women having NILM.

In an aspect, the presently disclosed subject matter provides a kit for triaging a human papillomavirus (HPV)-positive woman into colposcopy, the kit comprising: (a) a container containing primers and/or probes specific for a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM); and (b) instructions for use of the primers and/or probes in triaging a human papillomavirus (HPV)-positive woman into colposcopy.

Certain aspects of the presently disclosed subject matter having been stated hereinabove, which are addressed in whole or in part by the presently disclosed subject matter, other aspects will become evident as the description proceeds when taken in connection with the accompanying Examples and Figures as best described herein below.

BRIEF DESCRIPTION OF THE FIGURES

Having thus described the presently disclosed subject matter in general terms, reference will now be made to the accompanying Figures, which are not necessarily drawn to scale, and wherein:

5

FIG. 1A shows a description of the method used. Exemplary steps 1-9 as shown include liquid prep and or urine sample is collected and sent to the lab, DNA isolation, human and HPV DNA sequence capture, bisulfite treatment of human and HPV DNA (or enzymatic restriction with CG sensitive enzymes), sequencing, HPV
10 genotyping to identify high risk strains, human and HPV DNA methylation analyses to identify differentially methylated regions (DMRs), identify premalignant lesions/assess cervical cancer risk/assess risk of progression to CIN2+, and deliverables: cervical cancer screening and/or triage results. FIG. 1B shows the project workflow, for example, discovery was performed in cervical cancer biopsy
15 samples and cervical brush epithelium from normal controls. Nimblegen 385K CpG plus Promoter oligonucleotide arrays were used to hybridize samples enriched with Methylated DNA Immunoprecipitation (MeDIP). Promoter methylation of *ZNF516*, *INTS1* and *FKBP6* discriminated between normal and cancer samples. The panel was Validated on cervical brush samples from patents with Cervical Intraepithelial
20 Neoplasia 2 - 3 (CIN2+) compared to patents with No Intraepithelial Lesions or Malignancy (NILM) (n=211). Promoter methylation of *ZNF516*, *INTS1* and *FKBP6*, the host gene panel, together with HPV16-L1 DNA methylation discriminated between CIN2+ and NILM on liquid prep samples (n=67). Custom sequence capture probes were designed to capture the high-risk HPV genome and methylome, before
25 performing library prep for massively parallel Next-Generation Sequencing. The ccfdNA HPV16-L1 methylation is tested in urine, and plasma samples;

FIG. 2A shows ROC curves of *FKBP6* *INTS1* *ZNF516* promoter methylation, comparing CIN2-3 vs NILM in cervical brush samples from Chile (n=126). FIG. 2B shows ROC curve of a combined molecular panel integrated by *FKBP6* *INTS1*
30 *ZNF516* promoter methylation, comparing CIN2-3 vs NILM in cervical brush samples from Chile (n=126);

FIG. 3A shows ROC curves of *FKBP6*, *INTS1*, *ZNF516* promoter methylation and *HPV16-L1* methylation, comparing CIN2-3 vs NILM in liquid-based cytology

samples (n=67). FIG. 3B shows ROC curve of a combined molecular panel integrated by *FKBP6*, *INTS1*, *ZNF516* promoter methylation and HPV16-L1 methylation, comparing CIN2-3 vs NILM in liquid-based cytology samples. (n=67);

FIG. 4 shows amplification curves for the HPV ccfDNA-qPCR assay on
5 ccfDNA from women with Cervical Intraepithelial Neoplasia 1 (CIN1), Cervical Intraepithelial Neoplasia 2-3 (CIN2-3) and women with no Intraepithelial Lesions or Neoplasia (NILM). ccfDNA samples were analyzed for high risk HPV DNA using our HPV ccfDNA SYBR green qPCR assay. HeLa genomic DNA was included as a positive control. Ct ranges are variable, but are in a similar range as the genomic
10 control. NILM samples did not amplify;

FIG. 5 shows sequence capture of methylated high risk HPV.

FIG. 6A shows ROC curves for HPV16-L1 methylation and *ZNF516*, *FKBP6* and *INTS1* promoter methylation in plasma ccfDNA samples from patients diagnosed with CIN2+ and NILM, with area under ROC curve = 0.8075. FIG. 6B shows ROC
15 curves for HPV16-L1 methylation and *ZNF516*, *FKBP6* and *INTS1* promoter methylation in urine ccfDNA samples from patients diagnosed with CIN2+ and NILM, with area under ROC curve = 0.8611;

FIG. 7 shows a custom-designed pool of HPV-specific dual sequence capture probes was used for library amplification and target selection in ccfDNAs
20 (Roche/NimbleGen SeqCap EZ Choice Library). Using a SYBR green qPCR assay, an HPV E1 region common to thirteen high-risk HPV types was amplified;

FIG. 8 shows an amplification plot demonstrating the successful HPV amplification and enrichment obtained with dual sequence capture. Delta Ct values for HeLa (pink/red) and CSCC7 (purples) were 14.88 and 12.66 respectively. Based
25 on an estimated efficiency for the assay, the approximate fold enrichment was greater than 1700;

FIG. 9 shows Pre- and Post-Capture HPV qPCR results for five ccfDNA samples together with HeLa genomic control (dark blue) and no DNA controls (purple);

FIG. 10 shows a profiling of sample TrDNA-456 by tiered read mapping to various databases, the graph indicates the percentage of reads that map to thirteen (13)
30 HPV types, human, bacteria and unknown genomes after profiling reads of the clinical sample TrDNA-456 by tiered read mapping;

FIG. 11 shows box plots showing the distribution of CpG methylation levels per sample after aligning to HPV16 (left) and HPV16-L1 region (right). The CpG methylation median in the clinical samples is significantly higher than in the cell lines and higher in urine ccfDNA from the CIN3 than from the CIN1 clinical sample
5 (p<0.05), as expected;

FIG. 12 shows Scatter plots of HPV 16-L1 methylation in urine ccfDNA. HPV16-L1 qMSP methylation can discriminate bisulfite treated urine ccfDNA from patients with normal cytology (n=10) from women with dysplastic cytology and premalignant cervical lesions (ASCUS n=8; CIN1 n=3; CIN2+ n=3) with 100%
10 Sensitivity and Specificity;

FIG. 13 shows circulating cell-free Renal DNA (ccfDNA) isolation results in the table. The table shows the comparison between DNA concentration obtained by isolating DNA from urine from the same participants utilizing two different extraction methods: Phenol chloroform (PC) and ccfDNA isolation methods; the illustrations
15 shows a schematic of one concept of how ccfDNA enters the blood and urine of the subject;

FIG. 14 shows a table comprising dual sequence capture of high risk HPV in ccfDNA aligned to all high risk HPV types and low risk HPV types from PaVE database (top) and box plots of dual sequence capture of high risk HPV in ccfDNA
20 aligned to all high risk HPV types and low risk HPV types (bottom); and

FIG. 15 shows pairwise alignment of the HPV16 genome with reads obtained from urine ccfDNA from seven clinical samples: TrDNA-445, TrDNA-455, TrDNA-456, TrDNA-481, TrDNA-504, TrDNA-513, and TrDNA-571. The “close-up” HPV DNA computes and displays nucleotide-level (‘close-up’) multiple alignments of
25 sequences in a 1Kb region starting at a user-specified address or gene in the reference genome.

DETAILED DESCRIPTION

The presently disclosed subject matter now will be described more fully hereinafter with reference to the accompanying Figures, in which some, but not all
5 embodiments of the inventions are shown. Like numbers refer to like elements throughout. The presently disclosed subject matter may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Indeed, many modifications and other embodiments of
10 the presently disclosed subject matter set forth herein will come to mind to one skilled in the art to which the presently disclosed subject matter pertains having the benefit of the teachings presented in the foregoing descriptions and the associated Figures. Therefore, it is to be understood that the presently disclosed subject matter is not to be limited to the specific embodiments disclosed and that modifications and other
15 embodiments are intended to be included within the scope of the appended claims.

The presently disclosed subject matter provides methods and kits for triaging a human papillomavirus (HPV)-positive woman into colposcopy. It has been found that a group of genes exhibit increased promoter methylation in women having CIN2+
20 (NILM). The presently disclosed methods provide a triage test that can discriminate between those women with no intraepithelial lesions or malignancy (NILM) and women with abnormal cervical biopsies (CIN1, CIN2, CIN3), carcinoma in-situ, and cervical cancer.

Rapid advances in genomic technologies and TCGA have revealed extensive,
25 previously unsuspected complexity in human cancers. This complexity has led to calls for "precision medicine" to address the underlying complex genetic and epigenetic processes that underlie somatic cell evolution to malignancy. The present inventive methods take a significant step forward to improve precision medicine based on DNA sequence changes in methylation and mutation of the somatic genome
30 of the patient and HPV. The inventive methods are anchored in the phases of biomarker development as published by NCI EDRN several years ago. They also include cloud based approaches for generalized testing and implementation of early detection strategies. Specific strategies employed include DNA sequence based assays such as quantitative Methylation Specific PCR, bisulfite genomic sequencing,

and Next Generation Sequencing, all of which generate complex datasets reflecting the complexity of neoplastic evolution in human tissues. The inventive methods also propose testing urine samples using methods that optimize DNA isolation to enrich for cell free fragmented DNA (cffDNA) that is excreted in the urine, which opens a novel approach to early detection. The cloud based tools proposed in the methods could eventually be used by patients, providers, researchers and even insurance personnel. This type of "precision medicine" approach based on the actual DNA methylation and DNA nucleotide changes in the evolving virus and neoplasm of necessity vastly increase the complexity of the data.

10

I. METHODS FOR TRIAGING A HUMAN PAPILLOMAVIRUS (HPV)-POSITIVE WOMAN INTO COLPOSCOPY

In some embodiments, the presently disclosed subject matter provides a method for triaging a human papillomavirus (HPV)-positive woman into colposcopy, the method comprising: (a) selecting a HPV-positive woman testing positive for one or more high risk types of HPV; (b) obtaining nucleic acid from a test sample from the selected HPV-positive woman; (c) determining in the nucleic acid from the test sample of the selected HPV-positive woman a promoter methylation level of the promoter regions of a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM); and (d) triaging the HPV-positive woman into colposcopy when the level of promoter methylation of the group of genes is increased relative to the level of promoter methylation of the group of genes in a reference sample obtained from women having NILM.

As used herein, the term "colposcopy" refers to a medical procedure that uses a device, such as a magnifying device, to view the cervix of a woman. If a problem is seen during a colposcopy, a small tissue sample or biopsy can be taken from the cervix or from inside of the opening of the cervix. A goal of a colposcopy is to detect cervical dysplasia, such as intraepithelial lesions, premalignant cervical neoplasia lesions and/or cervical cancer lesions. As used herein, the term "trialoging" refers to the sorting of and/or allocation of treatment to patients, particularly those women at risk for cervical cancer.

As used herein, a human papillomavirus (HPV)-positive woman or a woman who tests positive for HPV is a woman who has been infected with HPV, particularly

a high risk type of HPV. The term “high-risk HPV” as used herein refers to those HPV types or strains that may progress to precancerous lesions and invasive cancer, such as cervical cancer. Non-limiting examples of high-risk HPV strains that are known or thought to cause cervical cancer include HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82. In some embodiments, one or more high risk types of HPV are selected from the group consisting of HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and HPV68. In some embodiments, a woman who has been infected with a high risk type of HPV is selected for triaging using the presently disclosed methods. In some embodiments, the HPV-positive woman is further selected on the basis of abnormal cytology, such as a woman who has cervical cells that do not appear normal in shape, size, and/or composition. In some embodiments, the abnormal cytology comprises low squamous intraepithelial lesions (LSIL). In some embodiments, the abnormal cytology comprises high squamous intraepithelial lesions (HSIL). In some embodiments, the HPV-positive woman does not have abnormal cytology of cervical cells. In some embodiments, abnormal cytology is tested for by using a Papanicolaou test (Pap-test). In some embodiments, the HPV-positive woman has had a negative, positive, or inconclusive Pap smear.

In some embodiments, selecting the HPV-positive woman testing positive for one or more high risk types of HPV comprises determining whether the nucleic acid is homologous to one or more high risk types of HPV. In some embodiments, determining whether the nucleic acid is homologous to one or more high risk types of HPV comprises performing at least one HPV detection assay selected from the group consisting of nucleic acid sequencing (e.g., Sanger sequencing, third generation sequencing, and the like), PCR, a HPV genotyping assay, a microarray assay, and a mRNA based assay. An example of a HPV detection assay is disclosed in PCT Patent Application No. PCT/US2014/019934, which is hereby incorporated by reference in its entirety. Methods to determine homology between two nucleic acid sequences are well known in the art and any method that can be used to determine homology can be used for the presently disclosed methods.

In some embodiments, determining whether the nucleic acid is homologous to one or more high risk types of HPV comprises: (a) sequencing the nucleic acid to produce a nucleotide sequence; (b) performing a sequence alignment between the nucleotide sequence and the nucleotide sequence of the one or more high risk types of

HPV; and (c) determining the percentage sequence identity between the nucleotide sequence and the nucleotide sequence of the one or more high risk types of HPV.

“Sequence identity” or “identity” in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window, and can take into consideration additions, deletions and substitutions. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (for example, charge or hydrophobicity) and therefore do not deleteriously change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have sequence similarity. Approaches for making this adjustment are well-known to those of skill in the art.

“Percentage of sequence identity” means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions, substitutions, or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions, substitutions, or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Algorithmic programs for sequence comparisons and/or searching sequences against databases are well known in the art, such as FASTA, BLAST, SAHA, MUMmer, AVID, CHAOS, QUASAR, and the like.

The terms “substantial identity” or “homologous” in their various grammatical forms in the context of polynucleotides means that a polynucleotide comprises a sequence that has a desired identity, for example, at least about 60% or more identity, such as 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%,

88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or higher identity compared to a reference sequence. Alignment programs using standard parameters can be used to determine percent identity. In some embodiments, at least a 60% sequence identity between the nucleotide sequence from the test sample and
5 the nucleotide sequence of the one or more high risk types of HPV means that at least one high risk HPV strain has been detected in the woman.

In some embodiments, nucleic acid is obtained from a test sample from the selected HPV-positive woman. Non-limiting examples of a test sample include a tissue specimen, a biopsy specimen, a surgical specimen, a cervical swab, a
10 cytological specimen, a plasma specimen, a serum specimen, and a urine specimen. In some embodiments, the test sample from the woman is from a specimen selected from the group consisting of a tissue specimen, a biopsy specimen, a surgical specimen, a cervical swab, a cytological specimen, a plasma specimen and a urine specimen. In some embodiments, the test sample from the woman comprises a specimen selected
15 from the group consisting of liquid prep, plasma, serum, and urine. As used herein, the term "liquid prep sample" refers to a sample that is in a fluid, such as a sample comprising cervical cells in a preservative liquid. Particularly, "liquid prep sample" can refer to a solution that comprises cervical cells that have been collected from a woman, such as by a tool (e.g., a brush, a self-collection swab, a tampon, and the
20 like). In some embodiments, the test sample refers to the liquid prep sample where the Pap and HPV test were performed, otherwise known as a reflex test. In some embodiments, the test sample refers to a self-collected cervical cytology or urine sample. Test samples will desirably contain cervical cells when they are collected from the cervical mucosa and/or circulating DNA from cervical cells, when it is
25 isolated from urine as ccfDNA.

As used herein, a "nucleic acid" or "polynucleotide" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs
30 thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-

stranded DNA found, inter alia, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

It may be beneficial to extract nucleic acids from the cells prior to testing. Some techniques of testing may not require pre-extraction. In some embodiments, as used herein, "obtaining" the nucleic acid from a test sample means using the nucleic acid in the test sample in an assay, such as a promoter methylation assay. In some embodiments, "obtaining" the nucleic acid means to separate the nucleic acid from other molecules found in the test sample, such as lipids, carbohydrates and proteins. Separating the nucleic acid may occur by any technique known in the art, for example, extraction with organic solvents, filtration, precipitation, absorption on solid matrices (e.g., silica resin, hydroxyapatite or ion exchange), affinity chromatography (e.g., via sequence specific capture or nucleic acid specific ligands), molecular exclusion chromatography, and the like. In some embodiments, the nucleic acid comprises DNA. In some embodiments, the nucleic acid comprises ccfDNA, which are relatively short, fragmented pieces of circulating DNA that get filtered by the kidneys and can be isolated from urine. In some embodiments, the nucleic acid is from about 150 to about 250 base pairs.

In some embodiments, after obtaining nucleic acid from the test sample from a woman, a promoter methylation level of the promoter regions of a group of genes is determined. It has been found that the promoter methylation level of the promoter regions of a group of genes exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM).

In some embodiments, the group of genes consists of, consists essentially of, or comprises a combination of at least three of the woman's endogenous genes. As used herein, the term "endogenous gene" of a woman is a gene that originated within the woman, as compared to a viral gene found in a sample taken from the woman, which did not originate within the woman. In some embodiments, the group of genes consists of, consists essentially of, or comprises *ZNF516* (e.g., NCBI Gene ID 9658), *FKBP6* (e.g., NCBI Gene ID 8468), and *INTS1* (e.g., NCBI Gene ID 26173).

In some embodiments, the group of genes consists of, consists essentially of, or comprises a combination of at least one of the woman's endogenous genes and at least one HPV gene. In some embodiments, the combination of at least one of the woman's endogenous genes and at least one HPV gene consists of, consists essentially of, or comprises *ZNF516*, *FKBP6*, and *INTS1* and HPV gene *HPV16-L1*.

Any tests can be used to detect promoter methylation. Suitable tests which can be used without limitation include lab-on-chip technology, microfluidic technologies, biomonitor technology, proton recognition technologies (e.g., Ion Torrent), single cell third generation sequencing (e.g. PacBio or Oxford Nanopore MinION hand-held sequencer; <http://phys.org/news/2015-06-professors-handheld-dna-sequencer.html>), and other highly parallel and/or deep sequencing methods. In some embodiments, determining the promoter methylation level comprises performing bisulfite modification to the nucleic acid from the test sample to produce a bisulfite modified nucleic acid. DNA methylation is a biochemical process whereby a methyl group is added to the cytosine or adenine DNA nucleotides. CG dinucleotides tend to cluster in regions called CpG islands, mainly present in the promoters of genes. Promoter methylation can directly switch off gene expression by preventing transcription factors binding to promoters.

Bisulfite compounds, for example, sodium bisulfite, convert non-methylated cytosine residues to bisulfite modified cytosine residues. The bisulfite ion treated gene sequence can be exposed to alkaline conditions, which convert bisulfite modified cytosine residues to uracil residues. Sodium bisulfite reacts readily with the 5,6-double bond of cytosine (but poorly with methylated cytosine) to form a sulfonated cytosine reaction intermediate that is susceptible to deamination, giving rise to a sulfonated uracil. The sulfonate group can be removed by exposure to alkaline conditions, resulting in the formation of uracil. The DNA can be amplified, for example, by PCR, and sequenced to determine whether CpG sites are methylated in the DNA of the sample. Uracil is recognized as a thymine by Taq polymerase and, upon PCR, the resultant product contains cytosine only at the position where 5-methylcytosine was present in the starting template DNA. One can compare the amount or distribution of uracil residues in the bisulfite ion treated gene sequence of the test cell with a similarly treated corresponding non-methylated gene sequence. A decrease in the amount or distribution of uracil residues in the gene from the test cell indicates methylation of cytosine residues in CpG dinucleotides in the gene of the test

cell. The amount or distribution of uracil residues also can be detected by contacting the bisulfite ion treated target gene sequence, following exposure to alkaline conditions, with an oligonucleotide that selectively hybridizes to a nucleotide sequence of the target gene that either contains uracil residues or that lacks uracil residues, but not both, and detecting selective hybridization (or the absence thereof) of the oligonucleotide. Examples of performing bisulfite modification to a nucleic acid to determine hypermethylation of the nucleic acid are disclosed in U.S. Patent No. 8,859,468, which is hereby incorporated by reference in its entirety. In some embodiments, the level of the bisulfite-modified nucleic acid is measured by sequencing of the bisulfite-modified nucleic acid.

In some embodiments, the level of the bisulfite modified nucleic acid is measured by quantitative real-time methylation specific PCR (QMSP). In general, PCR refers to an *in vitro* method for amplifying or replicating a specific polynucleotide template sequence. The PCR reaction involves a repetitive series of temperature cycles. The reaction mix usually comprises dNTPs (each of the four deoxynucleotides dATP, dCTP, dGTP, and dTTP), primers, buffers, DNA polymerase, and target nucleic acid molecule or template. The PCR step can use a variety of thermostable DNA-dependent DNA polymerases, such as Taq DNA polymerase, which has a 5'-3' nuclease activity but lacks a 3'-5' proofreading endonuclease activity. In real-time or quantitative PCR, the DNA is amplified and simultaneously quantified. QMSP protocols adopt the advantages of real-time PCR by using fluorescent-labeled MSP primers. Non-limiting examples of QMSP variations include Syber green-based QMSP, sensitive melting analysis after real-time MSP, and methylation-specific fluorescent amplicon generation.

In some embodiments, the quantitative real-time methylation specific PCR (QMSP) and/or sequencing of the bisulfite modified nucleic acid is performed using primers and/or probes specific for the group of genes. In some embodiments, a combination of the woman's endogenous genes consists of, consists essentially of, or comprises ZNF516, FKBP6, and *INTS1* and HPV gene HPV16. In some embodiments, the primers and/or probes are selected from the group consisting of SEQ ID NOS: 1- 12.

In some embodiments, the methylation status of the nucleic acid(s) of the one or more genes of interest are obtained by performing at least one human DNA methylation detection assay selected from the group consisting of nucleic acid

sequencing, PCR, a microarray assay, a restriction enzyme selection assay, a sequence capture assay, or an affinity enrichment assay.

In some other embodiments, the methylation status of the nucleic acid is obtained by performing at least one HPV DNA methylation detection assay selected
5 from the group consisting of nucleic acid sequencing, PCR, a microarray assay, a restriction enzyme selection assay, a sequence capture assay, or an affinity enrichment assay.

The terms "increased," "increase," "enhance," or "activate" are all used herein to generally mean an increase by a statically significant amount; for the avoidance of
10 any doubt, the terms "increased," "increase," "enhance," or "activate" means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as
15 compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level.

The power of a diagnostic test to correctly predict status is commonly measured as the sensitivity of the assay, the specificity of the assay or the area under a
20 receiver operated characteristic ("ROC") curve. Sensitivity is the percentage of true positives that are predicted by a test to be positive, while specificity is the percentage of true negatives that are predicted by a test to be negative. A ROC curve provides the sensitivity of a test as a function of 1-specificity. The greater the area under the ROC curve, the more powerful the predictive value of the test. Other useful measures
25 of the utility of a test are positive predictive value and negative predictive value. Positive predictive value is the percentage of people who test positive that are actually positive. Negative predictive value is the percentage of people who test negative that are actually negative. Diagnostic tests that use these biomarkers may show a ROC of at least about 0.6, at least about 0.7, at least about 0.8, or at least about 0.9.

30 The promoter methylation level is differentially present in women having CIN2+ lesions and women having no intraepithelial lesions or malignancy (NILM), and therefore, is useful in triaging a HPV-positive woman into colposcopy. In certain aspects, the promoter methylation level of a group of genes is measured in a test sample using the methods described herein and compared, for example, to predefined

promoter methylation levels and correlated to Cervical Intraepithelial Neoplasia (CIN) grade. In particular aspects, the measurement(s) may then be compared with a relevant diagnostic amount(s), cut-off(s), or multivariate model scores that distinguish a NILM grade from a CIN2+ grade. The diagnostic amount(s) represents a measured amount of a promoter methylation level of a group of genes above which or below which a woman is classified as having a particular CIN grade. As is well understood in the art, by adjusting the particular diagnostic cut-off(s) used in an assay, one can increase the sensitivity or specificity of the diagnostic assay. In particular aspects, the diagnostic cut-off can be determined, for example, by measuring the amount of promoter methylation levels of a group of genes in a statistically significant number of samples from women with the different CIN grades, and drawing the cut-off to suit the desired levels of specificity and sensitivity.

In some embodiments, the presently disclosed method has a specificity of at least 60 %. In some embodiments, the method has a sensitivity of at least 90 %. In some embodiments, the method has a positive predictive value (PPV) of at least 52%. In some embodiments, the method has a negative predictive value (NPV) of at least 90%.

In some embodiments, the group of genes consists of, consists essentially of, or comprises ZNF516, FKBP6, and *INTS1* and the specificity is at least 88%, the sensitivity is at least 88%, the positive predictive value (PPV) is at least 93%, and the negative predictive value (NPV) is at least 90%.

In some embodiments, the group of genes consists of, consists essentially of, or comprises ZNF516, FKBP6, and *INTS1* and HPV gene HPV16-L1, and the specificity is at least 60%, the sensitivity is at least 90%, the positive predictive value (PPV) is at least 52%, and the negative predictive value (NPV) is at least 93%.

In some embodiments, the presently disclosed methods further comprise enriching the nucleic acid from the test sample before determining the promoter methylation level. In some embodiments, enriching the nucleic acid from the test sample comprises: (a) preparing a library of the nucleic acid from the test sample; (b) amplifying the library using PCR to form a pre-capture PCR library; (c) hybridizing the pre-capture PCR library to a custom-designed pool of HPV-specific and human-specific capture probes to form a post-capture PCR library; (d) amplifying the post-capture PCR library to produce enriched nucleic acid; and (e) optionally repeating steps (c) and (d).

The term “enriching” as used herein means to purify or partially purify the molecule of interest. In some embodiments, the custom-designed pool of HPV-specific and human-specific capture probes is designed to capture most or all of the genomes of high-risk HPV-specific types. In some embodiments, the custom-

5 designed pool of HPV-specific and human-specific capture probes is designed to capture only some regions of the genotype-specific regions of at least one high-risk HPV genome, such as 1, 2, 3, 4, or 5 or more regions. In some embodiments, the custom-designed pool of HPV-specific and human-specific capture probes is designed to capture 2 to 3 regions of the HPV genome that distinguishes high-risk from low-

10 risk HPV types. In some embodiments, the custom-designed pool of HPV-specific and human-specific capture probes does not capture low-risk HPV types. In some embodiments, the custom-designed pool of HPV-specific and human-specific capture probes is designed to capture regions of the HPV16 genome. In some embodiments, the custom-designed pool of HPV-specific and human-specific capture probes is

15 designed to capture most or all of the genomes of the group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM). In some embodiments, the custom-designed pool of HPV-specific and human-specific capture probes is designed to capture most or all of the promoters of the group of genes that

20 exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM). In some embodiments, the custom-designed pool of HPV-specific and human-specific capture probes is designed to capture most or all of the promoters of *ZNF516*, *FKBP6*, and *INTS1*.

25 In some embodiments, the presently disclosed subject matter provides a method for triaging a human papillomavirus (HPV)-positive woman into colposcopy, the method comprising: (a) selecting a HPV-positive woman testing positive for one or more high risk types of HPV; (b) providing a kit that comprises the probes and/or primers needed for determining in the nucleic acid from a test sample of the selected

30 HPV-positive woman a promoter methylation level of the promoter regions of a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM);

(c) obtaining nucleic acid from the test sample from the selected HPV-positive woman; (d) determining the promoter methylation level in the nucleic acid from the test sample of the selected HPV-positive woman using the provided kit; and (e) triaging the HPV-positive woman into colposcopy when the level of promoter methylation of the group of genes is increased relative to the level of promoter methylation of the group of genes in a reference sample obtained from women having NILM. In some embodiments, the kit comprises the probes and/or primers needed for determining in the nucleic acid from the test sample of the selected HPV-positive woman the promoter methylation level of the promoter regions of the group of genes consisting of, consisting essentially of, or comprising *ZNF516*, *FKBP6*, and *INTS1* and HPV gene *HPV16-L1*.

In some embodiments, the presently disclosed subject matter further comprises informing the woman or a treating physician of the result of the method of triaging the human papillomavirus (HPV)-positive woman into colposcopy. In some embodiments, the presently disclosed methods further comprise performing a colposcopy. In some embodiments, the presently disclosed subject matter further comprises providing a prognosis regarding the development of cervical cancer based on the colposcopy performed. In some embodiments, for example if the presently disclosed methods are negative for promoter methylation, the methods further comprise recommending that the woman continue to be screened or retested at regular intervals, such as every six months, every year, every two years, every three years or more.

In some embodiments, the presently disclosed subject matter further comprises diagnosing the woman as having cervical pre-cancerous lesions and/or cervical cancer.

In some embodiments, the presently disclosed subject matter further comprises recommending treatment and/or treating the woman. Treatment may include removal of precancerous lesions; radiation treatment; surgery, such as a hysterectomy, removal of lymph nodes, a cone biopsy, and/or a trachelectomy; and/or chemotherapy, using for example a platinum-containing anti-cancer drug (e.g. cisplatin) and/or a topoisomerase inhibitor (e.g., topotecan). In some embodiments, the treatment is selected from the group consisting of removal of precancerous lesions, radiation treatment, surgery, and chemotherapy.

In some embodiments, the presently disclosed methods further comprise monitoring the efficacy of the treatment. Monitoring may occur by performing a Pap smear, performing a colposcopy, testing for the presence of a high risk type of HPV, and/or using the presently disclosed methods. In some embodiments, monitoring the efficacy of the treatment occurs by at least one method selected from the group consisting of performing a Pap smear, performing a colposcopy, testing for the presence of a high risk type of HPV, and determining a promoter methylation level of the promoter regions of a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM).

II. KITS FOR TRIAGING A HUMAN PAPILLOMAVIRUS (HPV)-POSITIVE WOMAN INTO COLPOSCOPY

The presently disclosed subject matter also relates to kits for practicing the methods of the invention. By "kit" is intended any article of manufacture (e.g., a package or a container) comprising primers and/or probes specific for a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM).

In some embodiments, the presently disclosed subject matter provides a kit for triaging a human papillomavirus (HPV)-positive woman into colposcopy, the kit comprising: (a) a container containing primers and/or probes specific for a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM); and (b) instructions for use of the primers and/or probes in triaging a human papillomavirus (HPV)-positive woman into colposcopy.

In some embodiments, the group of genes consists of, consists essentially of, or comprises *ZNF516*, *FKBP6*, and *INTS1*. In some embodiments, the group of genes consists of, consists essentially of, or comprises *ZNF516*, *FKBP6*, and *INTS1* and HPV gene *HPV16*. In some embodiments, the primers and/or probes are selected from the group consisting of SEQ ID NOS: 1- 12.

III. METHODS FOR TREATING CERVICAL CANCER

In some embodiments, the presently disclosed subject matter provides methods for treating cervical cancer. In some embodiments, the presently disclosed subject matter provides a method for treating cervical cancer, the method comprising: (a) selecting a HPV-positive woman testing positive for one or more high risk types of HPV; (b) providing a kit that comprises the probes and/or primers needed for determining in the nucleic acid from a test sample of the selected HPV-positive woman a promoter methylation level of the promoter regions of a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM); (c) obtaining nucleic acid from the test sample from the selected HPV-positive woman; (d) determining the promoter methylation level in the nucleic acid from the test sample of the selected HPV-positive woman using the provided kit; (e) triaging the HPV-positive woman into colposcopy when the level of promoter methylation of the group of genes is increased relative to the level of promoter methylation of the group of genes in a reference sample obtained from women having NILM; (f) performing a colposcopy; (g) diagnosing the woman as having cervical pre-cancerous lesions and/or cervical cancer; and (h) treating the woman for cervical cancer. Optionally, the presently disclosed methods include providing a prognosis regarding the development of cervical cancer based on the colposcopy performed.

Treatment of the woman for cervical cancer may include removal of precancerous lesions; radiation treatment; surgery, such as a hysterectomy, removal of lymph nodes, a cone biopsy, and/or a trachelectomy; and/or chemotherapy. Non-limiting therapeutic agents that can be used in the treatment of the woman for cervical cancer include platinum-containing anti-cancer drug (e.g., cisplatin, carboplatin), topoisomerase inhibitors (e.g., topotecan), tubulin targeting agents (e.g., paclitaxel), pyrimidine analogs (e.g., fluorouracil), nucleoside analogs (e.g., gemcitabine), anti-mitotic agents (e.g., docetaxel), and alkylating agents (e.g., cyclophosphamide).

Following long-standing patent law convention, the terms “a,” “an,” and “the” refer to “one or more” when used in this application, including the claims. Thus, for example, reference to “a subject” includes a plurality of subjects, unless the context clearly is to the contrary (e.g., a plurality of subjects), and so forth.

Throughout this specification and the claims, the terms “comprise,” “comprises,” and “comprising” are used in a non-exclusive sense, except where the context requires otherwise. Likewise, the term “include” and its grammatical variants

are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items.

For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing amounts, sizes, dimensions, proportions, shapes, formulations, parameters, percentages, parameters, quantities, characteristics, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term “about” even though the term “about” may not expressly appear with the value, amount or range. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are not and need not be exact, but may be approximate and/or larger or smaller as desired, reflecting tolerances, conversion factors, rounding off, measurement error and the like, and other factors known to those of skill in the art depending on the desired properties sought to be obtained by the presently disclosed subject matter. For example, the term “about,” when referring to a value can be meant to encompass variations of, in some embodiments, $\pm 100\%$ in some embodiments $\pm 50\%$, in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed methods or employ the disclosed compositions.

Further, the term “about” when used in connection with one or more numbers or numerical ranges, should be understood to refer to all such numbers, including all numbers in a range and modifies that range by extending the boundaries above and below the numerical values set forth. The recitation of numerical ranges by endpoints includes all numbers, e.g., whole integers, including fractions thereof, subsumed within that range (for example, the recitation of 1 to 5 includes 1, 2, 3, 4, and 5, as well as fractions thereof, e.g., 1.5, 2.25, 3.75, 4.1, and the like) and any range within that range.

EXAMPLES

The following Examples have been included to provide guidance to one of ordinary skill in the art for practicing representative embodiments of the presently disclosed subject matter. In light of the present disclosure and the general level of skill in the art, those of skill can appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications, and alterations can

be employed without departing from the scope of the presently disclosed subject matter. The synthetic descriptions and specific examples that follow are only intended for the purposes of illustration, and are not to be construed as limiting in any manner to make compounds of the disclosure by other methods.

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EXAMPLE 1

Human Papilloma Virus (HPV) testing is increasingly used for cervical cancer screening in conjunction with cervical cytology. Although privacy, cultural, and infrastructure issues challenge the effective implementation of HPV testing for cervical cancer screening worldwide, several countries have already implemented HPV testing in their screening protocols. There are currently no tests that can reliably identify the patients with abnormal cytology and positive oncogenic HPV results (HPV+) that need to be referred for colposcopy. A triage test from cytology to colposcopy needs to be able to discriminate in the cytology sample those patients with a Cervical Intraepithelial Neoplasia (CIN) grade more likely to progress to cervical cancer (CIN2+).

We set out to identify a panel of methylated human papilloma virus (HPV) and human genes that can discriminate between CIN2+ and normal/CIN1 patients in liquid prep samples and transrenal DNA isolated from urine (ccfDNA).

20 Methods: We used three independent cohorts, from Chile, Baltimore, and Puerto Rico, to develop a method that can be used to triage into colposcopy, HPV+ women with abnormal cytology. Participants were women with no cervical intraepithelial lesions or malignancy, and women with: abnormal cervical biopsies (CIN1, CIN2, CIN3); carcinoma *in-situ*, and cervical cancer.

25 Using DNA methylation arrays for Discovery and quantitative Methylation Specific PCR (qMSP) for Validation, we found that promoter methylation of *ZNF516*, *FKBP6*, and *INTS1* discriminates samples with CIN2+ lesions from samples with no intraepithelial lesions or malignancy (NILM): 88.3% sensitivity, 88.9% specificity, 93.2 Area Under the Curve (AUC), 86.9% positive predictive value (PPV) and 90.2% negative predictive value (NPV). Using custom sequence capture pools of baits, we pulled down genomic and bisulfite converted high-risk HPV DNA before library prep for NGS in 454 and MiSeq instruments, respectively. Using our NGS results, we optimized a Syber Green qPCR assay to detect high risk HPV DNA and a qMSP primer-probe set to detect methylated HPV. We replicated the results in liquid prep

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samples (n=67), adding HPV16 methylation to the panel: 90.9% sensitivity, 60.9% specificity, 90.1 (AUC), 52.6% positive predictive value (PPV) and 93.3% NPV. These results were verified in plasma DNA (AUC=80.7) and ccfDNA (AUC=86.1) isolated from a subset of patients who provided the liquid prep samples.

5 Our results suggest that our panel of viral and host gene methylation markers may be used as a reflex test in liquid prep to triage high risk HPV positive women into colposcopy, or as a screening and triage test in ccfDNA, in combination with our high risk HPV test. The presently disclosed methods can be used to triage into colposcopy, women with abnormal Pap smears and positive oncogenic HPV results
10 (HPV+) using host and viral DNA methylation markers in liquid prep and ccfDNA.

Results

DNA samples isolated from cervical brush (n=211), liquid cytology (n=107), serum(n=40) and ccfDNA (n=130) from women with normal cervical epithelium,
15 premalignant cervical neoplasia lesions and cervical cancer lesions were examined to test the performance of viral and host DNA methylation markers as classifiers for triage into colposcopy (Figures 1a-b). We first identified a panel of host DNA methylation markers associated with CIN2+ biopsy lesions using genome-wide DNA methylation arrays for Discovery and qMSP for Validation in cervical brush samples
20 from Chile. This classifier was previously associated with cervical cancer and abnormal cytology samples in the same cohort (23).

Cervical brush samples (n=211) were genotyped for HPV with the Reverse Line Blot assay. After removing samples without a definitive biopsy result, we examined the promoter methylation frequency of three genes (*ZNF516*, *INTS1* and
25 *FKBP6*) in cervical brush samples from women with normal (n=34), CIN1 (n=34), CIN2 (n=33) and CIN3 (n=20) and cervical cancer (n=90) pathology reports. We compared samples from women with no intraepithelial lesions or malignancy (NILM) and CIN1 lesions with samples from women with CIN2+ lesions, and found that: *ZNF516* has 91.7% Sensitivity, 27.4% Specificity and an AUC of 0.62; *FKBP6* has
30 92.4% Sensitivity, 46.8% Specificity and an AUC of 0.68; *INTS1* has 93.8% Sensitivity, 30.3% Specificity and an AUC of 0.57. We then compared samples from women with NILM to samples from women with CIN2+ lesions, we found that: *ZNF516* has 91.7% Sensitivity, 38.9% Specificity and an AUC of 0.76; *FKBP6* has 90.9% Sensitivity, 67.6% Specificity and an AUC of 0.86; *INTS1* has 90.7%

Sensitivity, 40.5% Specificity and an AUC of 0.62.

The panel of three classifiers (*ZNF516*, *INTS1* and *FKBP6*) has 90% Sensitivity, 88.9% Specificity, an AUC of 0.93, a Positive Predictive Value (PPV) of 93.1% and a Negative Predictive Value (NPV) of 84.2%, when comparing women with no intraepithelial lesions or malignancy (NILM) with women with CIN2+ lesions (Table 1 below, Figure 2a and 2b).

Table 1. Promoter methylation frequency in cervical brush samples

Table 1
Chile – cytobrush
n=211

Lesion comparison	Marker	Sensitivity %	Specificity %	AUC%	PPV%	NPV%
CIN2+ vs. NILM/CIN1	<i>ZNF516</i>	91.7	27.4	61.6		
	<i>FKBP6</i>	92.4	45.8	68.4		
	<i>INTS1</i>	93.8	30.3	56.9		
	3 gene panel	60	74.5	77	57.1	78.7
CIN2+ vs. NILM	<i>ZNF516</i>	91.7	38.9	76.5		
	<i>FKBP6</i>	90.9	67.6	85.6		
	<i>INTS1</i>	90.7	40.5	62.2		
	3 gene panel	88.3	88.9	93.2	86.8	90.2

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We then tested this panel of three classifiers in liquid-based cytology samples (n=67) from women in Puerto Rico, a subset of which had been previously tested for concordance of high risk HPV genotype between liquid prep and urine DNA (30). We compared samples from women with NILM and CIN1 lesions with samples from women with CIN2+ lesions and found that: *ZNF516* has 72.7% Sensitivity, 48.1% Specificity and an AUC of 0.63; *FKBP6* has 63.6% Sensitivity, 34.6% Specificity and an AUC of 0.50; *INTS1* has 91% Sensitivity, 35% Specificity and an AUC of 0.66. We also tested the performance of HPV16-L1 methylation, using previously designed primers and probes (39), and found it had 63.6% Sensitivity, 57.7% Specificity and an AUC of 0.54. Then we compared samples from women with NILM with samples from women with CIN2+ lesions, we found that: *ZNF516* has 63.6% Sensitivity, 17.4% Specificity and an AUC of 0.50; *FKBP6* has 63.6% Sensitivity, 39.1% Specificity and an AUC of 0.50; *INTS1* has 63.6% Sensitivity, 39.1% Specificity and an AUC of 0.47. We also tested the performance of *HPV16-L1* methylation and found it had 63.6% Sensitivity, 100% Specificity and an AUC of 0.79.

25

The panel of four classifiers in liquid prep samples, *ZNF516*, *INTS1*, *FKBP6* and *HPV16-L1* has 90.9% Sensitivity, 60.9% Specificity, an AUC of 0.90, a PPV of 52.6% and a NPV of 93.3% when comparing NILM with CIN2+ lesions (Table 2 below, Figure 3A and 3B).

5

Table 2. Promoter methylation frequency in liquid prep samples

UPR – Liquid prep
n=67

Lesion comparison	Marker	Sensitivity %	Specificity %	AUC %	PPV%	NPV%
CIN2+ vs. NILM/CIN1	<i>ZNF516</i>	72.7	48.1	63.5		
	<i>FKBP6</i>	63.6	34.6	49.8		
	<i>INTS1</i>	90.9	34.6	66.1		
	<i>HPV16-L1</i>	63.6	57.7	54.2		
	4 gene panel	90.9	46.15	73.1	28.3	96
CIN2+ vs. NILM	<i>ZNF516</i>	63.6	17.4	50		
	<i>FKBP6</i>	63.6	39.1	50.1		
	<i>INTS1</i>	63.6	39.1	46.6		
	<i>HPV16-L1</i>	63.6	100	78.7		
	4 gene panel	90.9	60.9	90.1	52.8	93.3

EXAMPLE 2

10 Sequencing the hrHPV genome in urine ccfDNA

To enable the testing of this four gene panel in ccfDNA, we optimized a previously published ccfDNA isolation method and compared it to the gold standard, phenol chloroform DNA extraction method (FIG. 13). We developed sequence capture methods and quantitative PCR assays to measure HPV high-risk types and methylated HPV CpGs in ccfDNA from women with and without cervical dysplasia.

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To examine the hrHPV genome in urine ccfDNA we developed custom dual-sequence capture baits (Figure 7), to enrich samples for hrHPV DNA from 12 high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59). To assess the enrichment efficiency of the dual sequence capture method we developed the hrHPV ccfDNA-qPCR assay with primers that amplify the HPV E1 region common to 13 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). We compared pre-capture and post-capture DNA from cervical cancer cell lines that harbor high risk HPV, HeLa (HPV18) and CSCC7 (HPV16). PicoGreen quantitation was performed on both pre-capture and post-capture DNA samples, and a normalized amount of DNA (5ng) was analyzed. FIG. 8 shows an amplification plot

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demonstrating the successful amplification and enrichment obtained with the dual sequence capture assay. Delta Ct values for HeLa and CSCC7 were 14.88 and 12.66, respectively. Based on an estimated efficiency for the assay, approximate fold enrichment was greater than 1700.

5 To assess the enrichment efficiency of the dual sequence capture method on clinical samples, we compared the HPV ccfDNA-qPCR assay results obtained on eight (8) pre-capture and post-capture HPV-enriched circulating DNA samples obtained from patients with CIN1 (n=4) and CIN2-3 (n=4). As shown in Figure 9, with data representing five clinical samples, the average Delta Ct value for Pre-
10 Capture vs. Post-Capture HPV-enriched ccfDNAs was 11.07, for an average fold enrichment of 670 of HPV ccfDNA in patient samples.

We then examined if the HPV ccfDNA-qPCR assay could discriminate ccfDNA isolated from women with and without cervical dysplasia. Figure 4 shows the amplification curves for the HPV ccfDNA-qPCR assay on ccfDNA from CIN2-3
15 (n=14), CIN1 (n=13) and 10 samples from women with NILM. The frequency of dysplastic samples that amplified differed significantly from NILM samples, but not with lesion severity: NILM (30%), CIN1 (77%, p=0.02), and CIN2+ (71%, p=0.04).

We then used massively parallel next generation sequencing to quantify the different HPV genotypes present in ccfDNA from 7 patients with CIN1 (n=3) and
20 CIN2-3 (n=4), using DNA from two cervical cancer cell lines (HeLa and CSCC7) as positive controls. The DNA was enriched for high risk HPV DNA with the custom dual sequence capture high risk HPV assay prior to multiplexed sequencing on a 454 GS Junior (Roche) system. The multiplexed massively parallel sequencing runs produced 230,385 reads with an average length of 138.3 bp. The reads in the clinical
25 samples covered 82-100% of the reference HPV 16 and 73-100% of the HPV 18 genomes, with an average of 21% (HPV18) to 33% (HPV18) percentage of all reads mapping to the sequences. These results were comparable to the percentage of all reads that mapped to the reference sequences in the positive control samples: 92% of the CSCC7 reads mapped on target covering 77% of the HPV16 genome; and 89% of
30 the HeLa reads mapped to 67% of HPV 18, as expected (Table 3).

Table 3. Dual sequence capture of high risk HPV in TrDNA

Dual Sequence capture of high risk HPV in TrDNA
aligned to HPV 16 and HPV 18

Sample ID	lesion	HPV	Input DNA (ng)	# Reads	HPV15ref			HPV18ref			
					unique match reads	% of all reads	% coverage	unique match reads	% of all reads	% coverage	
HeLa	cancer	18	250	51952	cell line with hpv18			46284	89.1	87.07	
CSCC7	cancer	16	250	48095	44262	92	76.6	cell line with hpv16			
TrDNA 445	CIN3	16, 45	60	25998	24670	95	100	722	3	73.4	
TrDNA 455	CIN1	18	100	55002	144	6.3	81.6	53075	96.5	100	
TrDNA 456	CIN1	33	100	45982	953	2.1	91.4	1647	3.6	74.3	
TrDNA 481	CIN3	16	55	18159	6700	37	100	1185	6.5	87.1	
TrDNA 504	CIN3	16	60	33218	11227	33.8	99.4	4624	13.9	100	
TrDNA 513	CIN1	16, 66	30	25242	5085	20.1	100	2202	8.7	100	
TrDNA 571	CIN3	16	100	26472	12229	46	100	3989	15.1	100	
Average				72	32868	8715	33	96	9635	21	91

To quantify the presence and HPV genotype composition of the patient samples, we first aligned the reads against the >170 human HPV genomes in the PapillomaVirus Episteme (PaVE) database, maintained by the NIH NIAID Samples TrDNA_445 and TrDNA_455 had more than 80% of their reads mapped to HPV 16 and HPV 18, however, the remaining five samples were somewhat split, mapping to both HPV 16 and 18 genotypes, with many not mapping to either (FIG. 14 and Table 4 below).

10

Table 4. Profiling of HPV TrDNA 454 reads by tiered mapping

READS	Human papillomavirus type
193	Human papillomavirus type 44, complete genome
24	Human papillomavirus type 61, complete genome
841	Human papillomavirus type 56 clone Qv24970, complete genome
243	Human papillomavirus type 45 isolate Qv31035, complete genome
231	Human papillomavirus type 72
109	Human papillomavirus type 11 isolate LZod45-11, complete genome
837	Human papillomavirus type 42 isolate Tj43-42, complete genome
54	Human papillomavirus type 53 isolate Tj43-53, complete genome
493	Human papillomavirus type 18 complete sequence
732	Human papillomavirus type 16, complete genome
421	Human papillomavirus type 68b, complete genome
226	Human papillomavirus type 6 complete genome, isolate CAC231
156	Human papillomavirus 39
330	Human papillomavirus type 31 isolate QV12357, complete genome
10060	Human papillomavirus type 33 isolate Qv34189, complete genome
564	Human papillomavirus type 35 isolate QV29782, complete genome
145	Human papillomavirus type 52 isolate Qv03594, complete genome
1533	Human papillomavirus type 58 isolate Z094, complete genome
184	Human papillomavirus type 81 complete genome
17645	Human papilloma virus type 59, complete viral genome
48	Human papillomavirus 54, complete genome
10913	Unmapped

To resolve the remaining reads, we searched the human genome using the program Bowtie (39), and then a local copy of the database of NCBI reference bacterial genomes, using BLAST (40). Following this tiered mapping approach, only a small number of reads were still mapping to unknown genomes, a small number of which (<50) were linker contaminants, while the others could potentially represent novel HPV genotypes or other viruses or bacteria (FIG. 14 and Table 4 above, representing sample TrDNA_456). Figures 4, 8 and 9 show the percentage of reads that align to different HPV types, human, bacteria and unknown genomes for this same sample, TrDNA_456. Table 4 lists the reads mapping to twenty-one (21) HPV types after profiling reads of the clinical sample TrDNA-456 by tiered read mapping. Figure 10 shows the percentage of reads that map to thirteen (13) HPV types, human, bacteria and unknown genomes for clinical sample TrDNA-456.

Cloud-based visualization tools of the HPV genome for personalized medicine

We used two Cloud-based tools, which allow the comparative visualization of HPV genomes uploaded by users against a reference genome of interest (43). Our

demonstration cloud-based servers show the alignment results of 11 hrHPV types, 9 low risk HPV types, and seven clinical samples (TrDNA-445, TrDNA-455, TrDNA-456, TrDNA-481, TrDNA-504, TrDNA-513, and TrDNA-571) against a reference genome (HPV16).

5 The large-scale HPV DNA server (<http://enterix.cbcb.umd.edu/enteric/enteric-hpv.html>) produces a graphical ‘large-scale’ view of the pairwise alignments of twenty HPV genomes against a reference genome (HPV16 in this demo), together with annotations of genome rearrangement events (Figure 14 top).

 The “close-up” HPV DNA server
10 (<http://enterix.cbcb.umd.edu/menteric/enteric-hpv.html>) computes and displays nucleotide-level (‘close-up’) multiple alignments of sequences in a 1Kb region starting at a user-specified address or gene in the reference genome (Figure 14 bottom).

15 **Sequencing the hrHPV epigenome in urine ccfDNA**

 To examine the hrHPV genome in urine ccfDNA we used the Sure Select Methyl-Seq Target Enrichment (Agilent) assay to enrich DNA samples from two HPV16 positive cervical cancer cell lines, CaSki (ATCC® CRL-1550™, 600
20 integrated HPV16 copies) and SiHa (ATCC® HTB35™, 2 integrated HPV16 copies); two HPV16 positive Head and Neck Squamous Cell Carcinoma cell lines, SCC-47 and SCC-90; and urine ccfDNA from two clinical samples: one from a patient with ASCUS and CIN1 (TrDNA47) and another one from a patient with HSIL and CIN3 (TrDNA50). Samples were PCR-amplified using sample-specific indexed
25 (“barcoding”) primers for multiplexed sequencing on a MiSeq (Illumina) system.

 Reads from the FASTQ files were aligned to all HPV types in the PAVE database and we performed CpG methylation analysis using Bismark, modified to analyze high-risk HPV genomes. The multiplexed massively parallel sequencing run produced 14,442,406 reads with a length of 100 bp. The percentage of all reads of the
30 HPV positive cervical cancer and head and neck cancer squamous cell carcinoma (HNSCC) cell lines that mapped uniquely to some of the reference genomes in the PAVE database can be seen in the top panel of (Table 5 below) Caski (93%); SiHa (13%); SCC-047 (68%) and SCC-090 (87%).

Table 5. Alignment of HPV genotype from the custom sequence capture method against the PapillomaVirus Episteme (PaVE) database

Sample	Reference_genome	Total_Reads	Reads_No_Alignment	Reads_Aligned_uniquely	Mapping_efficiency
Caski	HPV_pave_all	1648170	119552	1528618	93%
SiHa	HPV_pave_all	1838822	1597909	240913	13%
SCC-047	HPV_pave_all	2391854	759528	1632326	68%
SCC-090	HPV_pave_all	2806373	363874	2442499	87%
TrDNA-34(CIN1)	HPV_pave_all	3183371	3181520	1848	0%
TrDNA-50 (CIN3)	HPV_pave_all	2573816	2568941	4869	0%
Total		14442406	8591324	5851073	
Average		2407068	1431887	975179	44%
Caski	HPV-16		142622	1505548	91%
SiHa	HPV-16		1602013	236809	13%
SCC-047	HPV-16		814051	1577803	66%
SCC-090	HPV-16		399578	2406795	86%
TrDNA-34(CIN1)	HPV-16		3181642	1729	>1%
TrDNA-50 (CIN3)	HPV-16		2572738	1078	>1%
Total			8712644	5729762	
Average			1452107	954960	43%
Caski	HPV_16_L1		1356064	292106	18%
SiHa	HPV_16_L1		1791950	46872	3%
SCC-047	HPV_16_L1		2043910	347944	15%
SCC-090	HPV_16_L1		2375812	430561	15%
TrDNA-34(CIN1)	HPV_16_L1		3182952	419	>1%
TrDNA-50 (CIN3)	HPV_16_L1		2573606	210	>1%
Total			13324294	1118112	
Average			2220716	186352	9%

5 Towards personalized HPV methylation landscapes

The percentage of methylation across the HPV genome in all six samples was obtained with the Methylator Extractor module in Bismark. Scatterplots of the percentage of methylation by chromosomal location in the HPV genome for each of the six samples are shown in Figure 4. The percentage of methylation is shown on the Y-axis of the upper panel. The X-axis shows the chromosomal location along the HPV genome for both panels, including the promoters at positions 97 and 670 of the HPV genome. The upper panel of each plot represents the percentage of CpG methylation. The bottom panel of each plot represents the HPV genes and the Upper Regulatory Region (41).

The different HPV genomes that aligned to the six samples are shown in different colors and shapes (HPV16-black dots; HPV35 red squares; HPV52 red

triangles; and HPV71 green squares). The two cervical cancer cell lines had different patterns of CpG methylation. Caski had overall higher levels of methylation than SiHA for CpGs across the genome. SiHA exhibits a bimodal distribution of methylation percentage. The majority of CpGs below the 3500 position in the HPV genome have less than 60% methylation, while CpGs located between 3500 and 7200 positions on the HPV genome show over 80% methylation. This may be related to the low number of HPV copies per genome present in this cell line. The methylation patterns for both HPV positive HNSCC cell lines were very similar to those observed in Caski. The clinical samples aligned to more than one HPV type. TrDNA-34, a sample obtained from a patient with ASCUS and CIN1, aligned to HPV16 and HPV35. TrDNA-50, a sample obtained from a patient with HSIL and CIN3, aligned to HPV16, HPV52 and HPV71. The methylation patterns of HPV16 in both clinical samples are very similar to the methylation patterns observed in Caski and both HNSCC cell lines, albeit with less abundant number of reads, as expected.

15 Methylation of the remainder of HPV types was low overall.

To examine the HPV16 CpG methylation patterns we aligned the reads from the four cell lines to the HPV16 reference genome. The mapping efficiency (the percentage of total reads that aligned uniquely to the reference genome) to the HPV16 reference genome was very similar to the percentage of all reads that mapped to the PAVE reference database for the four cell lines: Caski (91%); SiHa (13%); SCC-047 (66%), and SCC-090 (86%). The mapping efficiency of the reads from the HNSCC cell lines to the HPV16 reference genome was in the range between the mapping efficiency obtained with SiHA and Caski, namely 86% for SCC-90 and 66% for SCC-47 (Table 5, middle panel). Since we know the number of copies of HPV16 DNA in SiHa (2) and Caski (~600), these sequencing results may be a good indicator of the number of HPV copies present in the HNSCC cell lines.

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The clinical samples aligned to more than one HPV type. TrDNA-34, a sample obtained from a patient with ASCUS and CIN1, aligned to HPV16 and HPV35. TrDNA-50, a sample obtained from a patient with HSIL and CIN3, aligned to HPV16, HPV52 and HPV71. The methylation patterns of HPV16 in both clinical samples are very similar to the methylation patterns observed in Caski and both HNSCC cell lines, albeit with less abundant number of reads, as expected.

30

Methylation of the remainder of HPV types was low overall.

Given that HPV type is defined by a measure of sequence divergence in the HPV L1 region of the genome, we divided the reads from the four cell lines that mapped only to the HPV16 L1 gene by the reads of the four cell lines that mapped to the HPV16 genome, to calculate their mapping efficiency to the HPV16-L1 gene.

5 The mapping efficiency of the four cell lines to the HPV-16 L1 gene was high: Caski (81%); SiHA (80%); SCC-47 (78%) and SCC-90 (82%). Surprisingly, the mapping efficiency of the clinical samples to the HPV16 L1 gene was as high as for the positive controls: 76% for TrDNA-34 and 81% for TrDNA-50 (Table 6).

10 Table 6. Mapping efficiency to HPV L1

Samples	Mapping_Efficiency to_HPVL1
Caski	81%
SCC-047	78%
SCC-090	82%
SiHa	80%
TrDNA-34ASi	76%
TrDNA-50	81%

We wanted to assess whether methylation levels in the HPV16-L1 variable region could be used as a marker of progression in cervical cancer premalignant lesions. To determine whether the L1 region is uniformly represented in the reads, we calculated the ratio of the reads from each of the four cell lines that mapped only to the HPV16 L1 gene (Table 5, bottom panel) to the total number of reads from each cell line that mapped to the HPV16 genome, and determined the mapping efficiency to the HPV16-L1 gene. The mapping efficiency of the four cell lines to the HPV-16 L1 gene was high: Caski (81%); SiHA (80%); SCC-47 (78%) and SCC-90 (82%). The mapping efficiency of the clinical samples to the HPV16 L1 gene was as high as for the positive controls: 76% for TrDNA34 and 81% for TrDNA50 (FIG. 11).

To further determine if HPV16-L1 methylation levels can be used as a surrogate marker of methylation of the HPV16 genome in urine ccfdNA, we examined the distribution of CpG methylation after aligning the urine ccfdNA samples to the HPV16-L1 gene. The CpG methylation median in the clinical samples

is significantly higher than in the cell lines and higher in urine ccfDNA from the CIN3 than from the CIN1 clinical sample ($p < 0.05$), as expected (Fig 11).

Quantification of viral and host DNA methylation in plasma and urine ccfDNA

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To enable the testing of this four-gene panel in urine ccfDNA, we optimized a previously published urine ccfDNA isolation method and compared it to the gold standard, phenol chloroform DNA extraction method (Figure 13, Top). We then designed primers and Taqman probes to quantify *HPV16 L1* DNA methylation and *ZNF516*, *INTS1* and *FKBP6* methylation in fragmented urine ccfDNA using qMSP. The genomic region of the *HPV16 L1* gene used to design the Primers were designed to amplify in urine ccfDNA short amplicons (80 base-pairs long) of the same genomic regions previously used to quantify *HPV16-L1* methylation in HNSCC(39); and *ZNF516*, *INTS1* and *FKBP6* in cervical cancer (18),

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In a feasibility study we found that *HPV16-L1* qMSP methylation can discriminate bisulfite treated urine ccfDNA from patients with normal cytology ($n=10$) from women with dysplastic cytology and premalignant cervical lesions (ASCUS $n=8$; CIN1 $n=3$; CIN2+ $n=3$) with 100% sensitivity and specificity (Figure 12).

15

We then quantified the methylation levels of the panel of viral and host DNA genes in plasma and urine ccfDNA samples from women with NILM and CIN2+ lesions. In plasma, we found the panel of four classifiers has 85.7% Sensitivity, 60.9% Specificity, an AUC of 0.807, PPV of 40% and a NPV of 93.3% (Figure 6a). In urine ccfDNA, we found the panel of four classifiers has 75% Sensitivity, 83.3% Specificity, an AUC of 0.861, PPV of 50% and a NPV of 93.8% (Figure 6b).

20

Discussion

The present inventors set out to identify a panel of methylated HPV and human host genes that can discriminate between CIN2+ and normal/CIN1 lesions in a reflex test performed in liquid prep samples and ccfDNA in plasma and urine. The inventors are the first to show that a panel of host and viral DNA methylation markers can discriminate between CIN2+ and NILM in multiple body compartments from the same individual: liquid prep, serum and urine. The results show that a precision

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medicine panel can be used as a reflex test in liquid prep to triage women referred to colposcopy. NGS reads from urine ccfDNA can be aligned in custom cloud-based servers for life-course personalized cervical cancer screening.

Women with low probability of having a CIN2+ lesion can be triaged out of a
5 biopsy after colposcopy. The four-gene classifier best performed in liquid-prep with Sensitivity (90.9%), Specificity of 60.9% and NPV (93.3%). In urine ccfDNA, the four-gene classifier had equal NPV (93.8%), a better Specificity (83.3%) and similar AUC (0.861). The results obtained for this classifier in liquid prep and urine ccfDNA warrant further study of this panel as a molecular biomarker to triage women referred
10 to colposcopy after testing positive for high-risk HPV and being diagnosed with cervical dysplasia with cytology. Women with low methylation values in this panel would be asked to return for follow-up cytology and HPV co-testing in 6-12 months, if the colposcopists do not see a clear indication of a lesion that should be biopsied. This would decrease the number of blind biopsies that are currently being performed,
15 decreasing screening costs and increasing health care quality.

The inventors have developed a method that can be used to triage women with HPV+ abnormal Pap smears who have been referred to colposcopy. Participants were women living in Chile and Puerto Rico with no cervical intraepithelial lesions or malignancy and women with abnormal cervical biopsies–CIN2+. The inventors used
20 methylation arrays during the Discovery phase, followed by bisulfite sequencing and quantitative Methylation Specific PCR (qMSP) during the Validation phase. Using custom sequence capture pools of baits, the inventors pulled down genomic and bisulfite converted high-risk HPV DNA before library prep for Next Generation Sequencing (NGS) in 454 and MiSeq instruments, respectively. Using the
25 methylation arrays results, they optimized a Syber Green qPCR assay to detect high risk HPV DNA and a qMSP primer-probe set to quantify promoter methylation of *ZNF516*, *FKBP6*, and *INTS1* in the host and the L1 gene in the HPV genome, in liquid prep and circulating cell free DNA (ccfDNA) in plasma and urine.

Additionally, we have quantified the circulating HPV methylome in urine,
30 using a custom sequence capture approach, which allows for multiplexed massively parallel sequence of clinical sample, followed by qMSP verification. Our results also show that qMSP quantification of *HPV16-L1* methylation is a surrogate of genome-wide HPV16 methylation, which could lead to high-throughput testing of *HPV16-L1* methylation by qMSP, digital PCR or multiplexed massively parallel sequencing.

Furthermore, the *HPV16-L1* methylation assay discriminates ccfDNA in urine from women with cervical dysplasia when compared to women that do not have cervical dysplasia.

Cervical cancer affects more than 1,000,000 women worldwide. Around
5 470,000 new cases of cervical cancer are detected annually, mostly in developing
nations, among which approximately half will die (42). Persistent mucosal infection
with an oncogenic (high risk) HPV genotype is the most significant cause of cervical
dysplasia and carcinoma (43). Only 14 of the genotypes are considered pathogenic or
high-risk (44). Multiple studies have linked genotypes 16, 18, 31, 33, 35, 39, 45, 51,
10 52, 56, 58, 59, 66, and 68 to disease progression (45). Patients with a persistent
infection with one of these types have an increased risk for developing severe
dysplasia or cervical carcinoma (46).

Co-testing with cytology and HPV at 5-year intervals is now the preferred or
acceptable strategy for cervical cancer screening for women aged 30-64 years in the
15 US. Clinical management for HPV-positive/Pap-negative women, however, is not
firmly established. In addition, there is increased resistance from the medical
community to accept HPV-PAP co-testing for cervical cancer, due to the complex
risk patterns associated to positive, negative, and undetermined cytology with
positive and negative HPV results.

Clinical detection of HPV is typically performed by *in-vitro* diagnostic assays
that detect viral genomic DNA, specifically the L1 gene, on mucosa samples collected
by cervical scraping. However, because HPV infections are very common and
because most women will clear HPV infections within 6 to 12 months, the presence of
HPV DNA does not mean that cervical dysplasia or cervical cancer is present or that
25 the infection will persist and the patient will progress to cervical cancer (13).

Furthermore, cervical cancer screening programs currently in use are inefficient at
identifying individuals at risk for disease, requiring multiple visits over a women's
lifetime, which is costly and cumbersome (47). New methods for cervical cancer
screening are needed to provide accurate, efficient and cost-effective ways of
30 identifying women at risk for cervical cancer.

Methods

Patient Samples

Cervical brush, liquid-based cytology, serum/plasma and urine samples were obtained from collaborators Chile and Puerto Rico, under the Johns Hopkins University School of Medicine Institutional Review Board (IRB) approved protocol #NA_00020633. The IRB of the Doctor Hernán Henríquez Aravena (HHA) tertiary care regional hospital, in Temuco, Chile and IRB of the University of Puerto Rico School of Medicine also approved this protocol.

Sample collection and processing

Retrospective cohort

10

DNA was isolated from the cervical epithelium and biopsy samples, genotyped for HPV with the Reverse Line Blot assay in Chile and sent to Johns Hopkins School of Medicine for epigenome-wide studies, as previously described (Epigenetics, 9, 308 (Feb 1, 2014)).

15

Prospective Cohort

Samples were collected, flash frozen and sent to Johns Hopkins School of Medicine for DNA extraction with phenol/chloroform method, bisulfite conversion NGS, qPCR and qMSP analyses as described below.

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Nucleic Acid extraction

Cervical cytobrush, liquid-based cytology, and plasma/serum samples (n=40) were centrifuged and their pellets were digested with 1% SDS and 20 µg/mL proteinase K (Sigma) at 48° C for 48 h, followed by phenol/chloroform extraction and ethanol precipitation.

25

Identification of methylation biomarkers of CIN2+ in liquid-based cytology

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We used quantitative Methylation Specific PCR (qMSP) to examine the association between CIN2+ biopsies and methylation of three genes (*ZNF516*, *FKBP6*, and *INTS1*) in cervical brush epithelium from a retrospective cohort, in which these genes are associated with cervical cancer and abnormal cytology (18). We verified the association between CIN2+ biopsies and methylation of *ZNF516*, *FKBP6*,

INTS1 and *HPV16-L1* in liquid-based cytology samples obtained from an independent prospective cohort.

Quantitative methylation-specific PCR (qMSP)

5

The methylation status of *ZNF516*, *FKBP6*, *INTS1* and *HPV16-L1* in bisulfite modified DNA from liquid-based cytology samples, plasma/serum and urine, was quantified with fluorescence-based quantitative methylation-specific PCR (qMSP) as described previously (Oncology Reports 32, 505 (Aug, 2014), Epigenetics, 9, (May 1, 10 2014)). Briefly, bisulfite converted DNA was used as template for fluorescence-based real-time PCR. Bisulfite sequencing (BS) was performed to determine the methylation status of the normal and tumor tissues prior to MSP. Bisulfite-treated DNA was amplified using BS primer sets for a 5' region within 800 bp of the TSS that included at least part of a CpG Island. The primer sequences did not contain CpG 15 dinucleotides in order to obtain unbiased sequencing PCR products. Each amplified DNA sample was sequenced using forward or reverse primers. After verifying with bisulfite sequencing that we had located a suitable area in the promoter region for qMSP validation, MSP primers and qMSP probes were designed to specifically amplify this region in the candidate gene promoters. Primers and probes were tested 20 on positive (in vitro methylated bisulfite converted DNA) and negative controls (genomic unmethylated bisulfite converted DNA) to ensure amplification of the desired product and non-amplification of unmethylated DNA, respectively. Primer and probe sequences are provided below.

Fluorogenic PCR reactions were performed in duplicates in a reaction volume 25 of 20 μ L that contained 3 μ L of bisulfite- modified DNA; 600 nM concentrations of forward and reverse primers; 200 nM probe; 0.6 U of platinum Taq polymerase (Invitrogen, Frederick, MD); 200 μ M concentrations each of dATP, dCTP, dGTP and dTTP; and 6.7 mM MgCl₂. Amplifications were performed using the reaction profile: 95 °C for 3 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min in a 30 7900HT sequence detector (Applied Biosystems) and were analyzed by a sequence detector system software (SDS 2.4; Applied Biosystems).

Primers and probes

Primers and probes sequences for the *HPV16-L1* gene region and for the ccfDNA amplicons isolated from ccfDNA are shown below.

HPV16-L1

- 5 F: TATAGCGGTTGGTTTGGGTTTGTG (SEQ ID NO: 1)
 R: ACATTCTCTATTATCCACACCTACA (SEQ ID NO: 2)
 Probe: /56-FAM/AGGTGTTGAGGTAGGTCGTGG-TAMRA-Sp (SEQ ID NO: 3)

ccfDNA primers and probes

- 10 (Probes are: 5'/56-FAM/-/ZEN/-/3IABkFQ/3')

FKBP6

- F: ATATTTTCGTATTTTATCGCG (SEQ ID NO: 4)
 R: ATCGTTTCGTTCCAACCG (SEQ ID NO: 5)
 15 Probe: CGACCCTAACCCCTCGCGAACTCTA (SEQ ID NO: 6)

ZNF516

- F: ACGGTGAGGTATGTATACG (SEQ ID NO: 7)
 R: ACTCGAAACCCTCAAAACG (SEQ ID NO: 8)
 20 Probe: AACGCCAAACCTCACCGTCGTACG (SEQ ID NO: 9)

INTS1

- F: CGTTTTTCGTCGTCGTTTTA (SEQ ID NO: 10)
 R: AAACAAAAAAATAACCGACGAT (SEQ ID NO: 11)
 25 Probe: TATAACCTCCGCCCTCCCTCCCTA (SEQ ID NO: 12)

Urine ccfDNA extraction and assessment

- We adsorbed cell-free nucleic acids, on a Q-Sepharose anion-exchange resin
 30 followed by a silica-based elution with LiCl, from urine samples (10 mL) provided by
 31 women with normal cervical cytology and 65 women with abnormal cervical
 cytology and biopsy: CIN1 (n=43) and CIN2-3 (n=22). ccfDNA samples were
 quantitated using the Quant-iT PicoGreen dsDNA reagent kit (Invitrogen, Life
 Technologies) and QuantiFluor ST spectrofluorometer (Promega). Concentrations

were calculated based on readings obtained from Lambda DNA standards. Quality (fragment size) assessment of the ccfDNA samples was performed by High Sensitivity DNA Lab Chip analysis on a BioAnalyzer 2100 (Agilent). The urine ccfDNA samples selected for further processing showed size profiles in the 50-300bp range, and exhibited low molecular weight DNA content.

ccfDNA hrHPV SYBR® Green Real-Time PCR

Validation of successful capture was assessed by the ccfDNA hrHPV qPCR assay on an Applied Biosystems Systems Prism 7500 Sequence Detection System. We designed SYBR® green quantitative PCR amplification assays of Beta-actin and the HPV E1 region that was used for the ccfDNA hrHPV capillary electrophoresis test (J. Clin. Microbiol. 52, 187 (Jan, 2014), J. Clin. Virol. (May 2, 2014)). This HPV E1 region is common to 13 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The resulting amplicon is 93 bp. Beta-actin (β -actin), a housekeeping gene, was used as an indicator for successful extraction of an equivalent length of viral DNA targets. Serial dilutions of DNA isolated from cervical carcinoma cell line CaSki (ATCC® CRL-1550™, 600 integrated HPV16 copies) were run in parallel as positive controls. Primers were obtained from Invitrogen (Carlsbad, CA).

Custom dual sequence capture assay

HPV-specific, biotinylated, long oligonucleotide probes were designed, synthesized, and pooled for target selection and enrichment utilizing a dual capture approach (Roche/NimbleGen SeqCap EZ Choice Library). Probes were designed to capture the complete HPV genome and well-characterized variants representing 12 clinically relevant high-risk HPV types that have been associated with cervical cancer (HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59 and 68b). The dual capture approach features two sequential captures of HPV target regions, with the output of the first capture amplified and used as targets for a second capture. The goal of this dual capture approach was a boost in both enrichment and specificity of HPV targets for deep sequencing.

Library preparation was performed using reagents in the GS FLX Titanium Rapid Library Preparation kit (Roche). To benchmark the assay we first worked with HPV positive cell lines: HeLa (HPV 18) and CSCC7 (HPV16). For these cell line samples, 250 ng genomic DNA was fragmented by nebulization, prior to ligation of double stranded, Rapid Library Multiplex Identifier (RL MID) adaptors, which added
5 unique 10 base pair sequence tags to each library enabling multiplexing for sequencing, according to standard Roche protocol.

Methods in the Roche Rapid Library preparation manual were adapted to work with the fragmented ccfDNA obtained from urine and optimized to prepare libraries
10 with as little as 30 nanograms of DNA (range =30-100 nanograms). Briefly, End-Polishing and A-tailing of fragments was performed, followed by ligation of the RL MID adaptors. Subsequently, two rounds of purification to remove un-ligated adaptors and adaptor dimers were performed on the fragments using AmPure XP beads according to standard Agencourt protocol. Quantitation of the library versus a
15 FAM-labeled standard was performed using a QuantiFluor ST spectrofluorometer (Promega). Quality assessment of the Library was performed by High Sensitivity DNA Lab Chip analysis on a BioAnalyzer 2100 (Agilent). A pre-capture amplification of the library was performed with ligation-mediated PCR (LM-PCR) for 12 cycles using primers complementary to the adaptors, followed by two rounds of
20 hybridization to the HPV-specific SeqCap EZ Choice Library. Each hybridization included enhancer oligonucleotides as well as Cot-1 Blocking DNA. After each hybridization step, a Streptavidin-coated, magnetic bead-based cleanup was performed, and the captured DNA was re-amplified by LM-PCR (5 cycles Post-Cap1, 15 cycles Post-Cap2). Subsequent to both Post-Cap LM-PCRs, purification was
25 performed using the QiaQuick PCR purification kit (Qiagen). After Post-Cap2 LM-PCR, HPV-enriched ccfDNAs were quantitated by PicoGreen fluorescent assay and quality was assessed by DNA High Sensitivity Lab Chip analysis on the BioAnalyzer 2100.

A calculation of fold- enrichment was based on Ct values of captured vs. non-
30 captured LM-PCR products in comparison with positive and negative controls. The amplified, HPV-enriched cell line DNAs and ccfDNAs were then diluted to a normalized concentration of 1×10^8 molecules per microliter, pooled and processed for multiplexed sequencing on the GS Junior system (Roche).

Validation of successful capture was assessed by a XEN-HPV qPCR Sybr green assay on an Applied Biosystems Systems 7500 Sequence Detection System. A calculation of fold- enrichment was based on Ct values of captured vs. non-captured LM-PCR products in comparison with positive and negative controls. The amplified, 5 HPV-enriched cell line DNAs and ccfDNAs were then diluted to a normalized concentration of 1xE08 molecules per microliter, pooled and processed for multiplexed sequencing on the GS Junior system (Roche).

RT-PCR assay

10 A Perkin-Elmer/ABI 7900 thermocycler was used to run SYBR green quantitative PCR amplification assays for β -actin and the HPV *E1* region that was used for the ccfDNA HPV capillary electrophoresis test. This ccfDNA HPV qPCR assay amplifies an HPV *E1* region common to 13 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The resulting amplicon is 93 bp. 15 Primers were obtained from Invitrogen (Carlsbad, CA).

Sequencing on the GS Junior system

Processing for sequencing was according to Lib-L emPCR Amplification Method and Sequencing Method manuals, GS Titanium Series (Roche). A 20 modification of reduced amplification primer (1:10 dilution) in the emPCR step was performed due to the small fragment size range. The DNA copy (fragment) to bead ratio for emPCR was 0.5. Sequence runs were performed on the GS Junior System.

Following completion of the sequencing runs, signal processing was performed, followed by QC analyses. Roche GS RunBrowser software deconvoluted 25 the data, assigned each read to the appropriate library, and was used to assess QC metrics (% Keypass wells, % pass filtering, Average Read length, and Total bases). GS Reference Mapper, from the Roche GS Analysis Software Suite, was used for initial alignment to the HPV 16 and HPV 18 reference genomes. Detailed analysis was performed as described below.

30

Bioinformatics analyses for GS Junior output reads.

To determine the HPV genotype composition of the patient samples, we searched the GS Junior 454 reads from each ccfDNA sample against the non-redundant database of HPV reference types compiled from the twelve high-risk and

nine low-risk HPV reference genomes and the larger collection of PapillomaVirus Episteme (PAVE) genome database(48). Sequences were aligned to the reference genome database with the software sim4db(49), retaining only the best alignment covering 50% or more of the query sequence. To resolve the remaining reads, we first searched the human genome with the program bowtie(39), allowing for partial matches (option '--local'), and then the database of NCBI bacterial reference genomes, using the tool blast(40). Following this tiered mapping approach, only a small number of reads were still unclassified, of which a small number (<100) were linker contaminants, whereas the others could potentially represent novel HPV genotypes and other viruses or bacteria.

Custom methylated HPV sequence capture and sequencing

To quantify the HPV methylome by HPV type in ccfDNA we developed a methylated HPV sequence method (HPV ccfDNA Meth-seq) using custom designed baits (Table 5). The Sure Select Methyl-Seq Target Enrichment (Agilent) workflow, developed to capture the Human Methylome with a starting DNA amount of 3 µg, was optimized to capture the HPV methylome of 12 high-risk HPV types that have been associated with cervical cancer (HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59 and 68b). Briefly, the Sure Select Methyl-Seq Library Prep Kit was used for End-Polishing and A-tailing of sheared DNA (150-200 bp) fragments, and subsequent ligation of methylated adaptors. These libraries were hybridized to target enrichment RNA baits in the custom SureSelect HPV Methyl-Seq bait library and hybrids were bound to streptavidin beads for enrichment. The target enriched gDNA library was then bisulfite treated and subsequently amplified by PCR and index tagged by a second PCR. The indexed, bisulfate-treated and enriched library was pooled, diluted to 2 nM, and submitted to the JHMI Synthesis and Sequencing Facility for sequencing on the Illumina MiSeq system. The library was denatured using 0.2 N NaOH and diluted to a loading concentration of 15 pM. Sequencing was performed using a 2 x 150 MiSeq Reagent Kit v2 (300 cycle) with the run performed as a 2 x 100 paired-end run. The sample was loaded with a 5% PhiX control spike-in to account for the low diversity of the sample library. FASTQ files were generated and analyzed as follows.

Analysis of Sequencing Data

FASTQC version 0.11.3 was used for quality control of all the paired-end reads to assess per sequence base quality, per tile sequence quality, per sequence quality scores, per base sequence content, per sequence GC content, per base N
5 content, sequence length distribution, sequence duplication levels, overrepresented sequences, adapter and kmer content. Reads were trimmed using Trim Galore v0.3.7. Default parameters were used, and one base pair was trimmed off at the end of all paired-end reads to improve paired-end mapping. If adapter contamination was observed, the standard Illumina adapters were trimmed off at the end of all paired-end
10 reads. FASTQC post-trimming was rerun to perform quality control to ensure sure that the trimming step did not produce any adverse side effects.

Bioinformatics analyses of bisulfite-converted reads

15 Briefly, for alignment purposes Bismark converts all C's to T's (in forward reads) and all G's to A's (in reverse reads) prior to mapping and maps these in silico converted reads, to both a C-to-T and G-to-A in silico-converted genome. After successful alignment it replaces the T's and A's back to their original bases in all converted reads and compares it to the original reference genome to deduce
20 methylated cytosines. Default parameters were used with the exception that "bowtie2 and 1 mismatch" was allowed during the alignment, retaining only the unique matches (default) and a seed length of 32. PCR duplicates were removed from the mapped reads using the "de-duplicate Bismark" routine. After running Bismark, post-alignment quality control was performed using Samtools version 0.1.19 and BamUtil
25 version 1.0.12. Bismark divides all cytosines into four categories: cytosines followed by guanines (CpGs), cytosines followed by non-guanines followed by guanines (CHGs), cytosines followed by at least two non-guanines (CHHs), and cytosines followed by N's (CNs). Analysis for the current study focused on CpGs. The Default Bismark methylation extractor routine was used with the exception of --paired-end, --
30 no-overlap, and minimum coverage of at least 1 read to extract all CpGs in individual samples.

Sequence similarity among HPV reference genomes could potentially impact the analysis, by disqualifying reads that map to several genomes. To eliminate or reduce these effects, we employed a two stage mapping procedure: i) we first aligned

the reads against the entire genome database, using the unique matches to select those genotypes represented in the samples, and then ii) re-aligned the reads against the subset of genomes that were detected in that sample. Methylation maps for the HPV16 genome and restricted to the L1 region were produced with data produced by
5 the Methylation Extractor from the Bismark suite.

REFERENCES

All publications, patent applications, patents, and other references mentioned in the specification are indicative of the level of those skilled in the art to which the
10 presently disclosed subject matter pertains. All publications, patent applications, patents, and other references are herein incorporated by reference to the same extent as if each individual publication, patent application, patent, and other reference was specifically and individually indicated to be incorporated by reference. It will be understood that, although a number of patent applications, patents, and other
15 references are referred to herein, such reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art. In case of a conflict between the specification and any of the incorporated references, the specification (including any amendments thereof, which may be based on an incorporated reference), shall control. Standard art-accepted meanings of terms are
20 used herein unless indicated otherwise. Standard abbreviations for various terms are used herein.

In case of a conflict between the specification and any of the incorporated references, the specification (including any amendments thereof, which may be based on an incorporated reference), shall control. Standard art-accepted meanings of terms
25 are used herein unless indicated otherwise. Standard abbreviations for various terms are used herein.

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20

THAT WHICH IS CLAIMED:

1. A kit for triaging a human papillomavirus (HPV)-positive woman into colposcopy, the kit comprising:
 - 5 (a) a container containing primers and/or probes specific for a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM), wherein the group of genes comprises *ZNF516*, *FKBP6*, and *INTS1* and HPV gene *HPV16-L1*; and
 - 10 (b) instructions for use of the primers and/or probes needed for determining in the nucleic acid from a test sample of the selected HPV-positive woman the promoter methylation level of the promoter regions of the group of genes comprising *ZNF516*, *FKBP6*, and *INTS1* and HPV gene *HPV16-L1* from the sample from a human papillomavirus (HPV)-positive woman comprising ccfDNA, wherein the primers
 - 15 and/or probes are suitable for use in a qMSP assay.
2. The kit of claim 1, wherein the primers and/or probes are selected from the group consisting of SEQ ID NOS: 1- 12.
- 20 3. The kit of claims 1 or 2, wherein the test sample from the woman of step b) is from a specimen selected from the group consisting of a tissue specimen, a biopsy specimen, a surgical specimen, a cervical swab, a cytological specimen, a plasma specimen, a serum specimen, and a urine specimen.
- 25 4. Use of the kit of claim 1 for triaging a human papillomavirus (HPV)-positive woman into colposcopy comprising:
 - (a) determining the promoter methylation level methylation level of the promoter regions of the group of genes comprising *ZNF516*, *FKBP6*, and *INTS1* and HPV gene *HPV16-L1* in the nucleic acid from the test sample of the selected HPV-
 - 30 positive woman using the provided kit; and
 - (b) triaging the HPV-positive woman into colposcopy when the level of promoter methylation of the group of genes is increased relative to the level of promoter methylation of the group of genes in a reference sample obtained from women having NILM.

5. The use of claim 4, wherein the HPV-positive woman is positive for one or more high risk types of HPV selected from the group consisting of HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and
5 HPV68
6. The use of claim 4, wherein the group of genes consists of, consists essentially of, or comprises a combination of at least three of the woman's endogenous genes.
- 10 7. The use of claim 4, wherein the group of genes consists of, consists essentially of, or comprises a combination of at least one of the woman's endogenous genes and at least one HPV gene.
8. The use of claim 4, wherein the test sample from the woman of step a) is from
15 a specimen selected from the group consisting of a tissue specimen, a biopsy specimen, a surgical specimen, a cervical swab, a cytological specimen, a plasma specimen, a serum specimen, and a urine specimen.
9. The use of claim 4, wherein the nucleic acid comprises DNA.
20
10. The use of claim 4, wherein the nucleic acid comprises ccfDNA.
11. The use of claim 4, wherein the nucleic acid is from about 150 to about 250 base
25 pairs.
12. The use of claim 4, wherein determining the promoter methylation level comprises performing bisulfite modification to the nucleic acid from the test sample to produce a bisulfite modified nucleic acid.
- 30 13. The use of claim 4, wherein the level of the bisulfite modified nucleic acid is measured by quantitative real-time methylation specific PCR (QMSP).
14. The use of claim 4, wherein the level of the bisulfite modified nucleic acid is measured by sequencing of the bisulfite modified nucleic acid.

15. The use of claim 4, wherein the quantitative real-time methylation specific PCR (QMSP) and/or sequencing of the bisulfite modified nucleic acid is performed using primers and/or probes specific for the group of genes.
- 5
16. The use of claim 4, wherein the primers and/or probes are selected from the group consisting of SEQ ID NOS: 1- 12.
17. A method for triaging a human papillomavirus (HPV)-positive woman into colposcopy, the method comprising:
- 10
- (a) selecting a HPV-positive woman testing positive for one or more high risk types of HPV;
 - (b) obtaining nucleic acid from a test sample from the selected HPV-positive woman;
 - 15 (c) determining in the nucleic acid from the test sample of the selected HPV-positive woman a promoter methylation level of the promoter regions of a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM); and
 - (d) triaging the HPV-positive woman into colposcopy when the level of
 - 20 promoter methylation of the group of genes is increased relative to the level of promoter methylation of the group of genes in a reference sample obtained from women having NILM.
18. A method for triaging a human papillomavirus (HPV)-positive woman into colposcopy, the method comprising:
- 25
- (a) selecting a HPV-positive woman testing positive for one or more high risk types of HPV;
 - (b) providing a kit that comprises the probes and/or primers needed for determining in the nucleic acid from a test sample of the selected HPV-positive woman
 - 30 a promoter methylation level of the promoter regions of a group of genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM);
 - (c) obtaining nucleic acid from the test sample from the selected HPV-positive woman;

(d) determining the promoter methylation level in the nucleic acid from the test sample of the selected HPV-positive woman using the provided kit; and

(e) triaging the HPV-positive woman into colposcopy when the level of promoter methylation of the group of genes is increased relative to the level of promoter methylation of the group of genes in a reference sample obtained from women having
5 NILM.

19. The method of claim 18, wherein the kit comprises the probes and/or primers needed for determining in the nucleic acid from the test sample of the selected HPV-
10 positive woman the promoter methylation level of the promoter regions of the group of genes consisting of, consisting essentially of, or comprising *ZNF516*, *FKBP6*, and *INTS1* and HPV gene *HPV16-L1*.

20. The method of any one of claims 17 to 19, wherein selecting the HPV-positive
15 woman testing positive for one or more high risk types of HPV comprises determining whether the nucleic acid is homologous to one or more high risk types of HPV.

21. The method of any one of claims 17 to 20, wherein determining whether the nucleic acid is homologous to one or more high risk types of HPV comprises
20 performing at least one HPV detection assay selected from the group consisting of nucleic acid sequencing, PCR, a HPV genotyping assay, a microarray assay, and a mRNA based assay.

22. The method of any one of claims 17 to 21, wherein determining whether the
25 nucleic acid is homologous to one or more high risk types of HPV comprises:

(a) sequencing the nucleic acid to produce a nucleotide sequence;

(b) performing a sequence alignment between the nucleotide sequence and the nucleotide sequence of the one or more high risk types of HPV; and

(c) determining the percentage sequence identity between the nucleotide
30 sequence and the nucleotide sequence of the one or more high risk types of HPV.

23. The method of any one of claims 17 to 22, wherein the one or more high risk types of HPV are selected from the group consisting of HPV16, HPV18, HPV31,

HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and HPV68.

24. The method of any one of claims 17 to 23, wherein the group of genes consists
5 of, consists essentially of, or comprises a combination of at least three of the woman's endogenous genes.

25. The method of any one of claims 17 to 24, wherein the group of genes consists
10 of, consists essentially of, or comprises *ZNF516*, *FKBP6*, and *INTS1*.

26. The method of any one of claims 17 to 25, wherein the group of genes consists
of, consists essentially of, or comprises a combination of at least one of the woman's endogenous genes and at least one HPV gene.

15 27. The method of any one of claims 17 to 26, wherein the combination of at least one of the woman's endogenous genes and at least one HPV gene consists of, consists essentially of, or comprises *ZNF516*, *FKBP6*, and *INTS1* and HPV gene *HPV16-L1*.

28. The method of any one of claims 17 to 27, wherein the test sample from the
20 woman of step b) is from a specimen selected from the group consisting of a tissue specimen, a biopsy specimen, a surgical specimen, a cervical swab, a cytological specimen, a plasma specimen, a serum specimen, and a urine specimen.

29. The method of any one of claims 17 to 28, wherein the test sample from the
25 woman of step b) comprises a specimen selected from the group consisting of liquid prep, plasma, serum, and urine.

30. The method of any one of claims 17 to 29, wherein the nucleic acid comprises
30 DNA.

31. The method of any one of claims 17 to 29, wherein the nucleic acid comprises TrDNA.

32. The method of any one of claims 17 to 30, wherein the nucleic acid is from about 150 to about 250 base pairs.
33. The method of any one of claims 17 to 32, wherein the HPV-positive woman is
5 further selected on the basis of abnormal cytology.
34. The method of any one of claims 17 to 35, wherein the HPV-positive woman has had a negative, positive, or inconclusive Pap smear.
- 10 36. The method of any one of claims 17 to 36, wherein determining the promoter methylation level comprises performing bisulfite modification to the nucleic acid from the test sample to produce a bisulfite modified nucleic acid.
37. The method of claim 36, wherein the level of the bisulfite modified nucleic acid
15 is measured by quantitative real-time methylation specific PCR (QMSP).
38. The method of claim 36, wherein the level of the bisulfite modified nucleic acid is measured by sequencing of the bisulfite modified nucleic acid.
- 20 39. The method of any one of claims 37 and 38, wherein the quantitative real-time methylation specific PCR (QMSP) and/or sequencing of the bisulfite modified nucleic acid is performed using primers and/or probes specific for the group of genes.
40. The method of claim 39, wherein the primers and/or probes are selected from
25 the group consisting of SEQ ID NOS: 1- 12.
41. The method of any one of claims 17 to 40, wherein the method has a specificity of at least 60 %.
- 30 42. The method of any one of claims 17 to 40, wherein the method has a sensitivity of at least 90 %.
43. The method of any one of claims 17 to 41, wherein the method has a positive predictive value (PPV) of at least 52%.

44. The method of any one of claims 17 to 42, wherein the method has a negative predictive value (NPV) of at least 90%.
- 5 45. The method of any one of claims 17 to 43, wherein the group of genes consists of, consists essentially of, or comprises *ZNF516*, *FKBP6*, and *INTS1* and the specificity is at least 88%, the sensitivity is at least 88%, the positive predictive value (PPV) is at least 93%, and the negative predictive value (NPV) is at least 90%.
- 10 46. The method of any one of claims 17 to 44, wherein the group of genes consists of, consists essentially of, or comprises *ZNF516*, *FKBP6*, and *INTS1* and HPV gene *HPV16*, and the specificity is at least 60%, the sensitivity is at least 90%, the positive predictive value (PPV) is at least 52%, and the negative predictive value (NPV) is at least 93%.
- 15 47. The method of any one of claims 17 to 45, further comprising enriching the nucleic acid from the test sample before determining the promoter methylation level.
48. The method of claim 47, wherein enriching the nucleic acid from the test sample
20 comprises:
- (a) preparing a library of the nucleic acid from the test sample;
 - (b) amplifying the library using PCR to form a pre-capture PCR library;
 - (c) hybridizing the pre-capture PCR library to a custom-designed pool of HPV-specific and human-specific capture probes to form a post-capture PCR library;
 - 25 (d) amplifying the post-capture PCR library to produce enriched nucleic acid;
- and
- (e) optionally repeating steps (c) and (d).
49. The method of any one of claims 17 to 48, further comprising informing the
30 woman or a treating physician of the result of the method of triaging the human papillomavirus (HPV)-positive woman into colposcopy.
50. The method of any one of claims 17 to 49, further comprising performing a colposcopy.

51. The method of claim 50, further comprising providing a prognosis regarding the development of cervical cancer based on the colposcopy performed.
- 5 52. The method of any one of claims 17 to 51, further comprising diagnosing the woman as having cervical pre-cancerous lesions and/or cervical cancer.
53. The method of any one of claims 17 to 52, further comprising recommending that the woman continue to be screened or retested at regular intervals.
- 10 54. The method of any one of claims 17 to 53, further comprising recommending treatment and/or treating the woman.
55. The method of claim 54, wherein the treatment is selected from the group
15 consisting of removal of precancerous lesions, radiation treatment, surgery, and chemotherapy.
56. The method of claim 55, further comprising monitoring the efficacy of the treatment.
- 20 57. The method of claim 58, wherein monitoring the efficacy of the treatment occurs by at least one method selected from the group consisting of performing a Pap smear, performing a colposcopy, testing for the presence of a high risk type of HPV, and determining a promoter methylation level of the promoter regions of a group of
25 genes that exhibit increased promoter methylation in women having CIN2+ lesions as compared to women having no intraepithelial lesions or malignancy (NILM).
58. The method any one of claims 17 to 53, wherein determining whether the methylation status of the nucleic acid is obtained by performing at least one human
30 DNA methylation detection assay selected from the group consisting of nucleic acid sequencing, PCR, a microarray assay, a restriction enzyme selection assay, a sequence capture assay, or an affinity enrichment assay.

59. The method of any one of claims 17 to 53, wherein determining whether the methylation status of the nucleic acid is obtained by performing at least one HPV DNA methylation detection assay selected from the group consisting of nucleic acid sequencing, PCR, a microarray assay, a restriction enzyme selection assay, a sequence
5 capture assay, or an affinity enrichment assay.

SCREENING WITH QUANTITATIVE METHYLATION SPECIFIC PCR (qMSP)

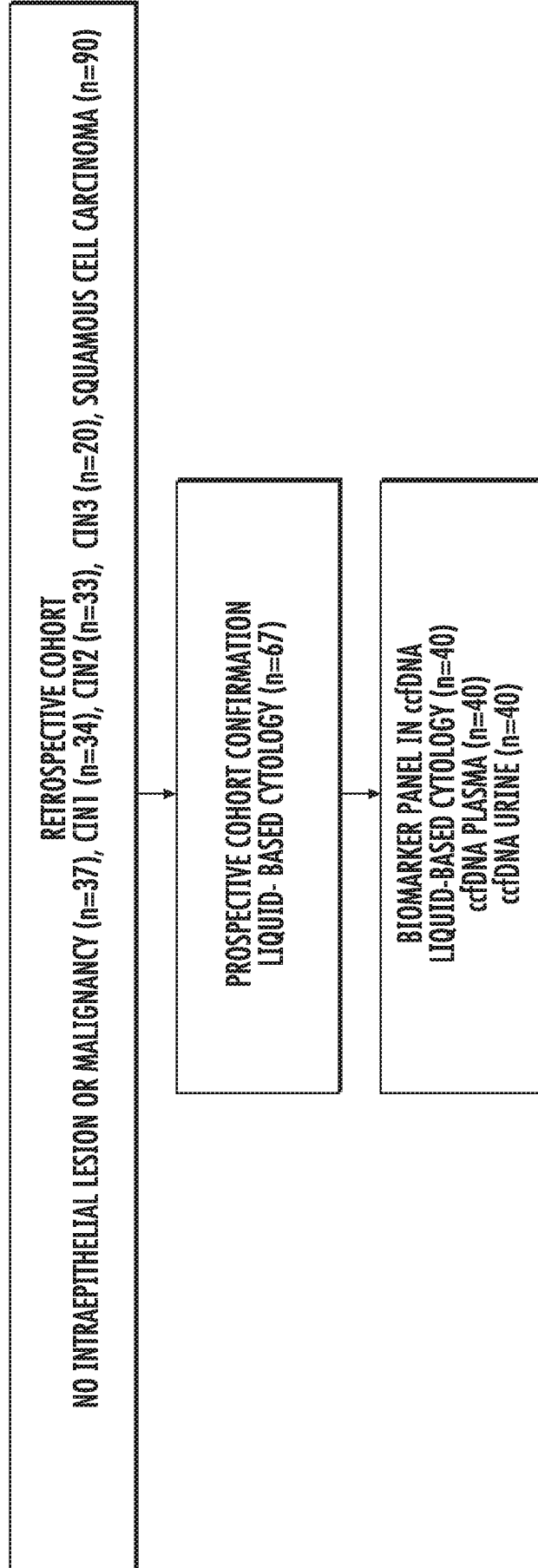


FIG. 1A

METHOD TO INTEGRATE NEXT GENERATION SEQUENCING AND qMSP FOR
CERVICAL CANCER SCREENING AND TRIAGE INTO BIOPSY

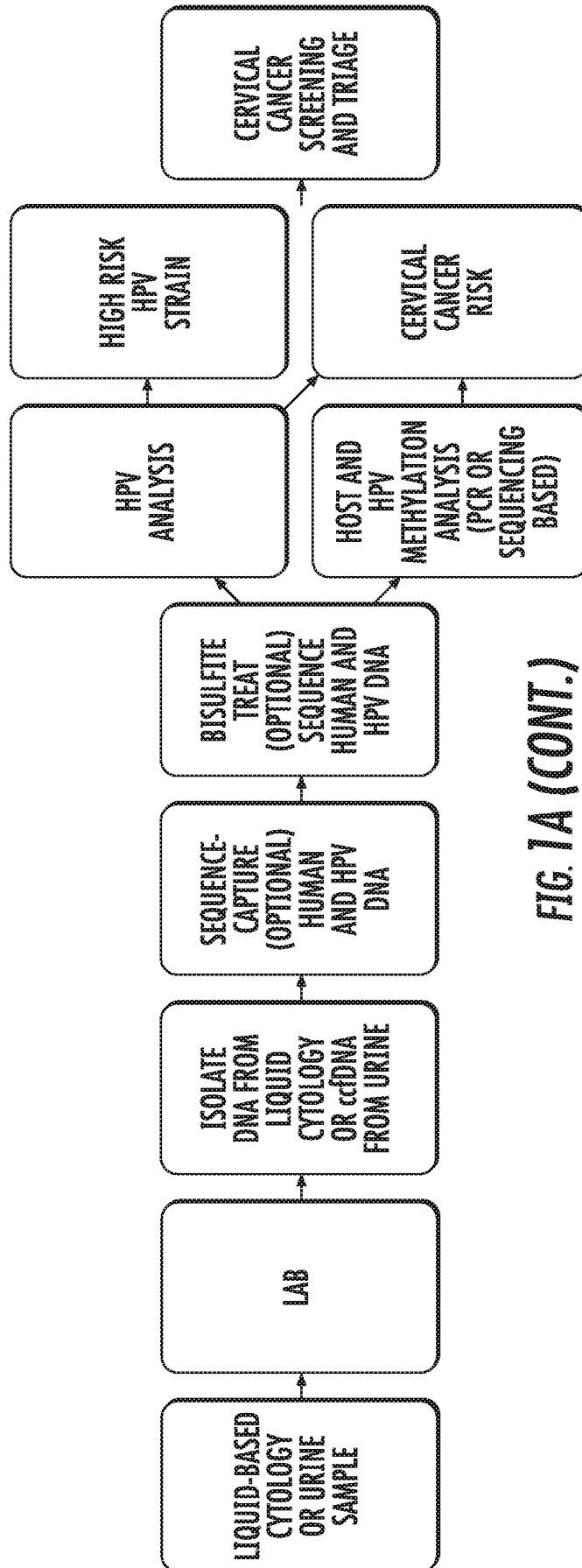


FIG. 1A (CONT.)

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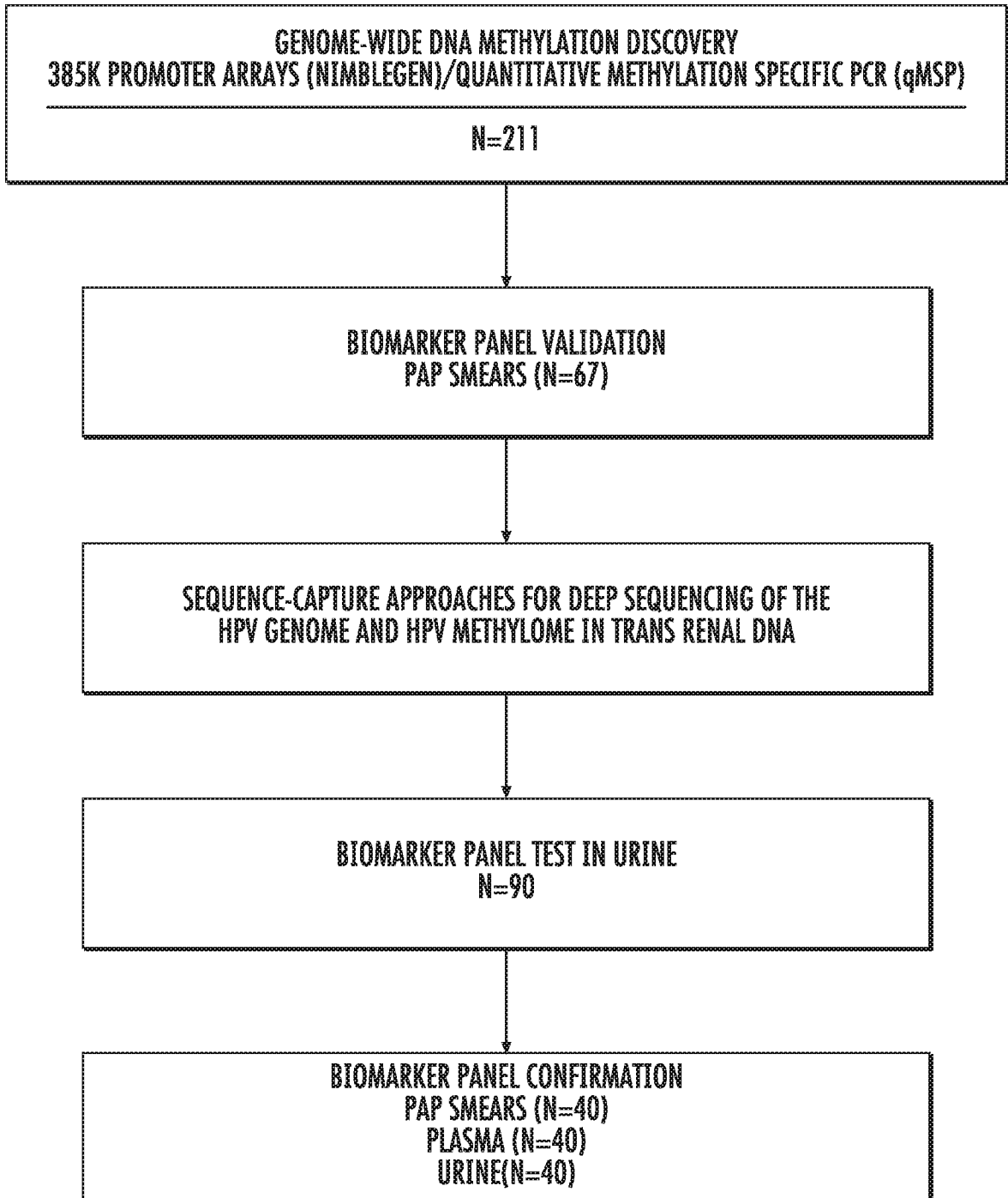


FIG. 1B

CIN2-3 vs NILM - FKBP6 INTS1 ZNF516
CHILE - CYTOBRUSH -ROC COMPARISON
n=126

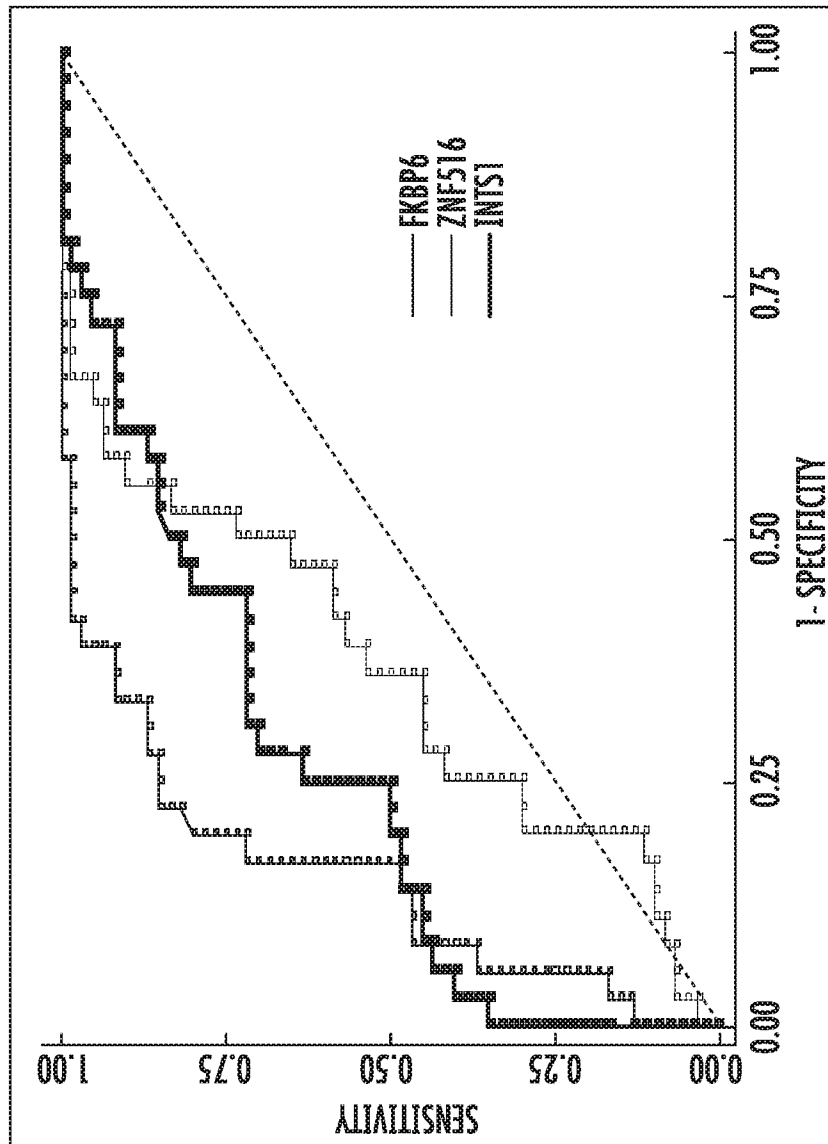


FIG. 2A

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CIN2-3 vs NILM - FKBP6, INTS1, ZNF516
CHILE - CYTOBRUSH - 3 GENE PANEL
n=126

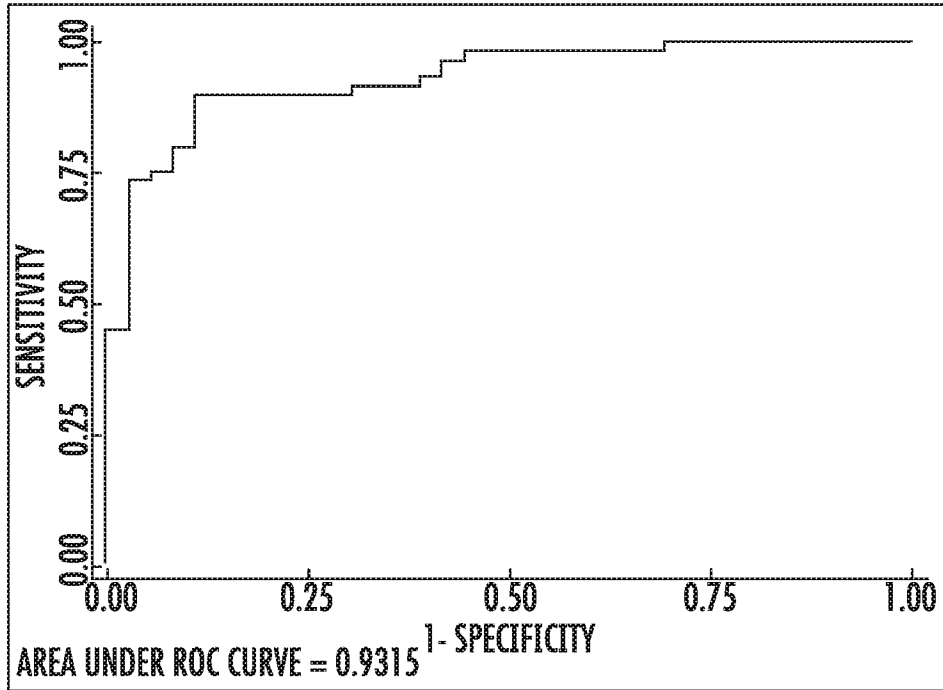


FIG. 2B

CIN2-3 vs NILM/CIN1 - FKBP6 INTS1 ZNF516 HPV16-L1
UPR - LIQUID PREP - ROC COMPARISON
n=67

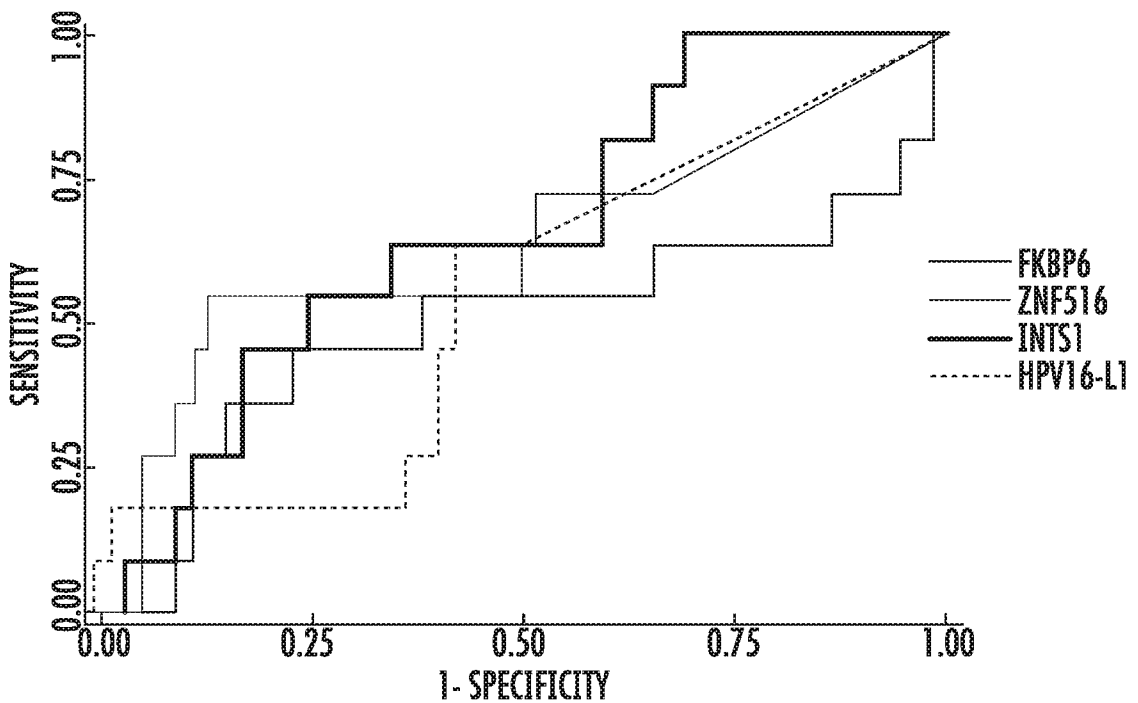


FIG. 3A

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CIN2-3 vs NILM - FKBP6 INTS1 ZNF516 HPV16-L1
UPR - LIQUID PREP -4 GENE PANEL
n=67

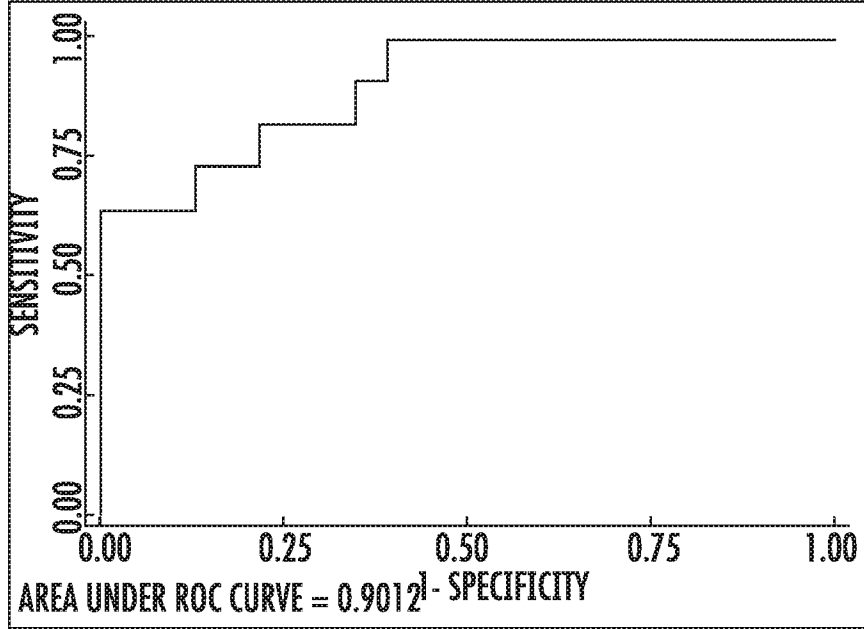
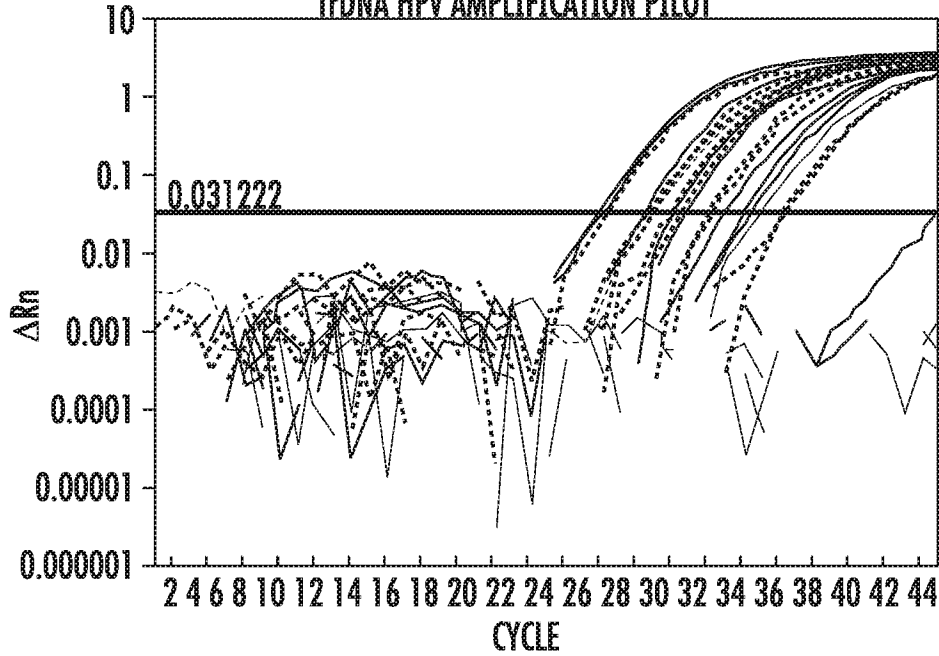


FIG. 3B

HIGH RISK HPV qPCR IN TrDNA

TrDNA HPV AMPLIFICATION PILOT



|CIN2 |NEG. CONTROL |NILM |CIN1 |HPV + CELL LINE CONTROL (HeLa)

FIG. 4

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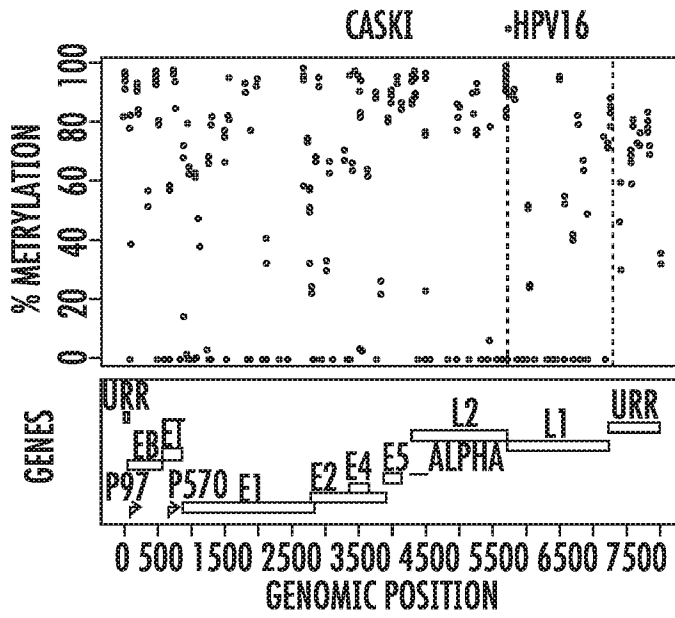


FIG. 5A

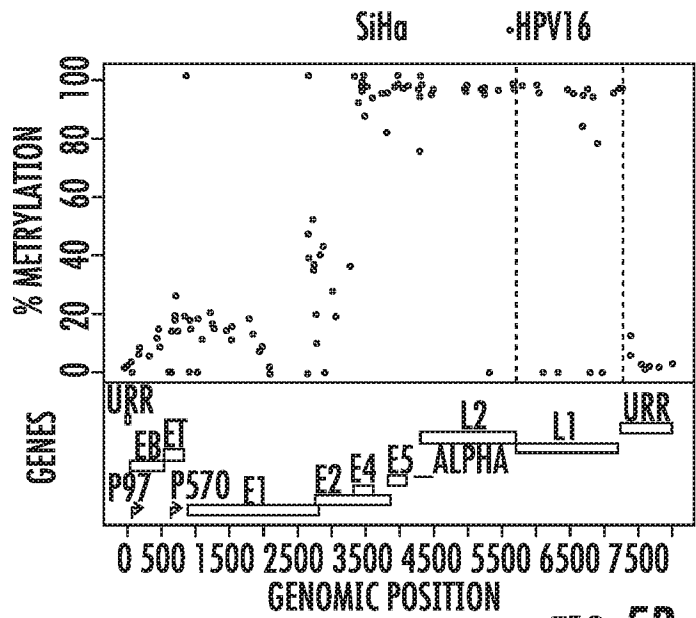


FIG. 5B

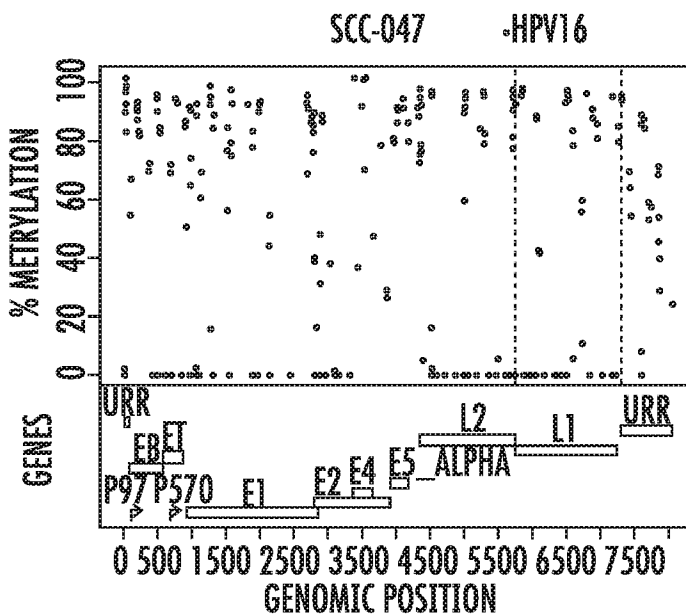


FIG. 5C

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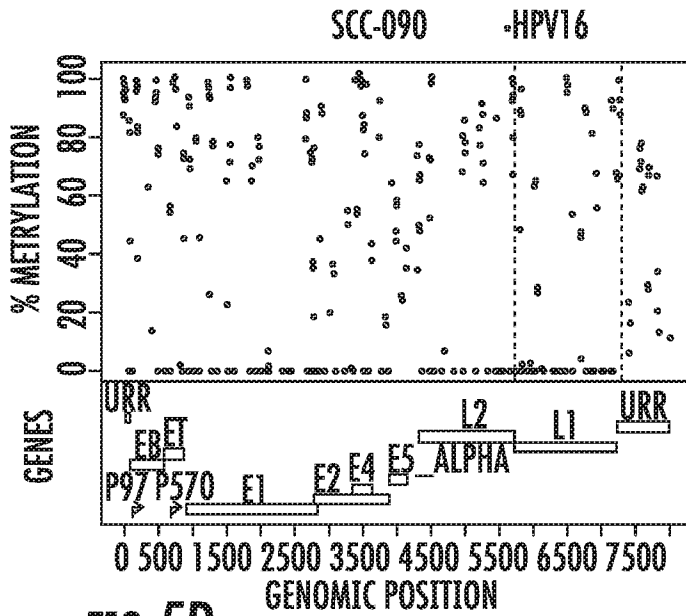


FIG. 5D

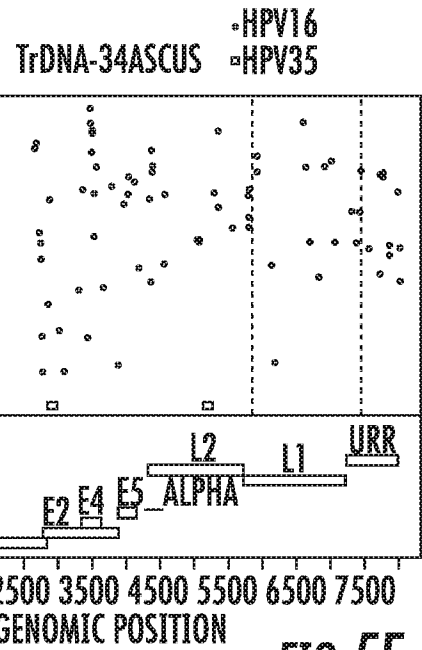


FIG. 5E

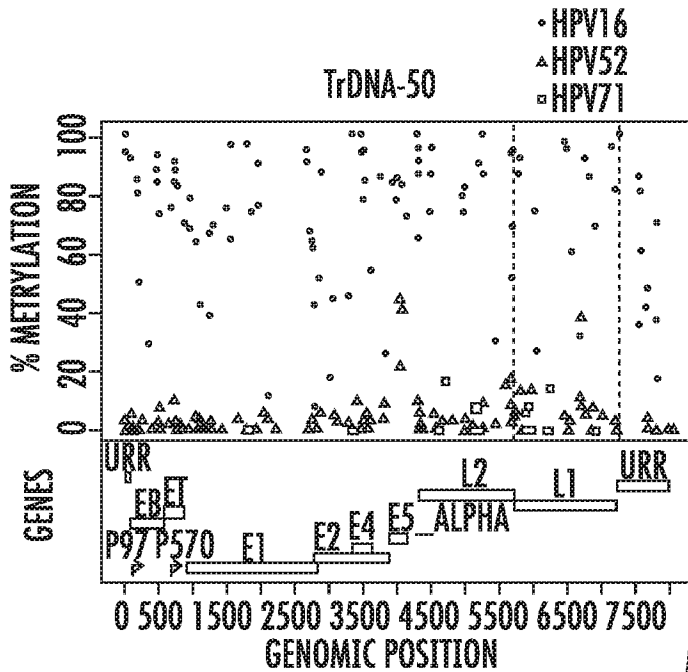


FIG. 5F

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CIN2n 3 vs NILM - FKBP6 INTS1 ZNF516 HPV16B L1
UPR - PLASMA - 4 GENE PANEL
n=30

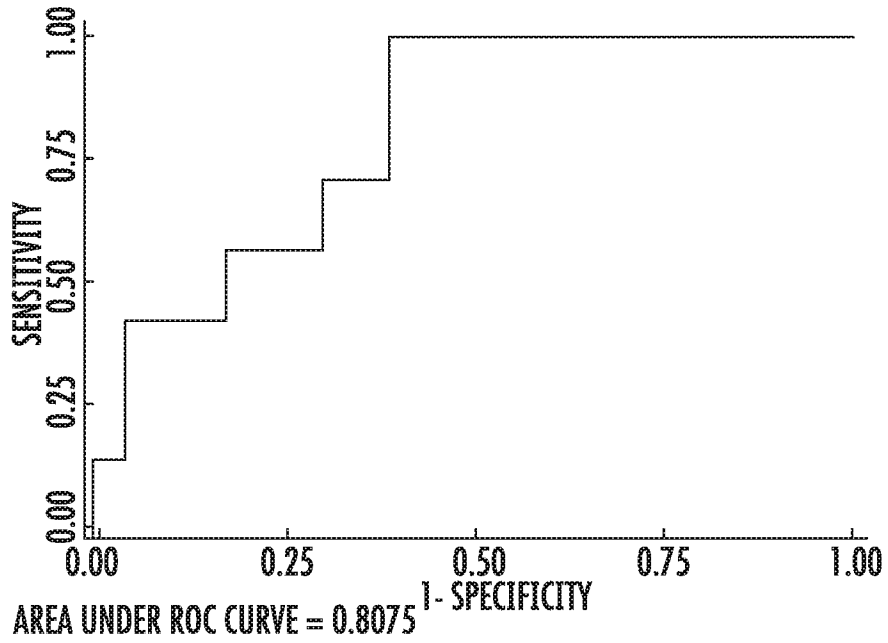


FIG. 6A

CIN2n 3 vs NILM - FKBP6 INTS1 ZNF516
HPV16B L1
UPR - URINE - 4 GENE PANEL
n=30

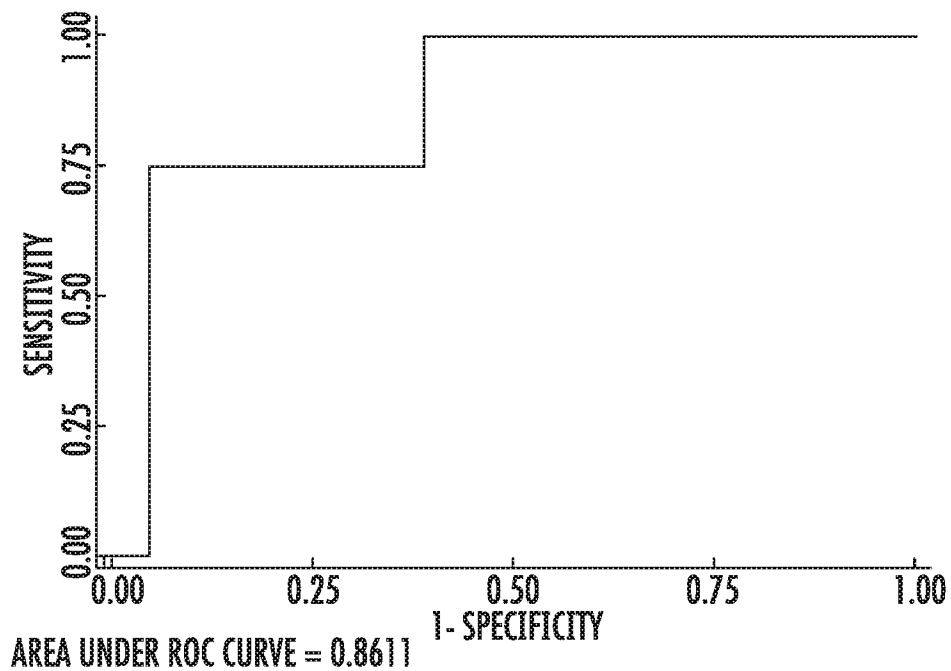


FIG. 6B

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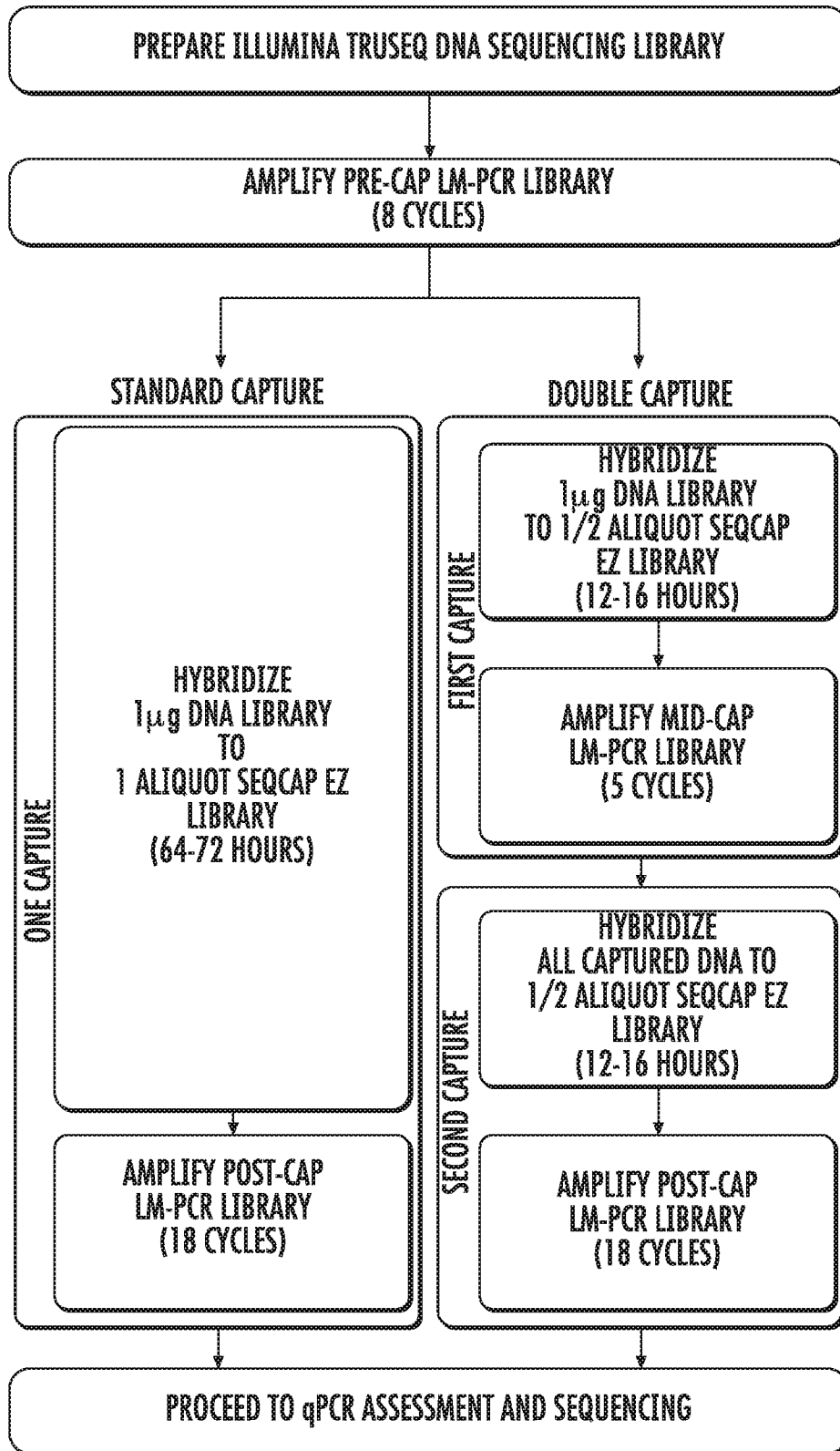


FIG. 7

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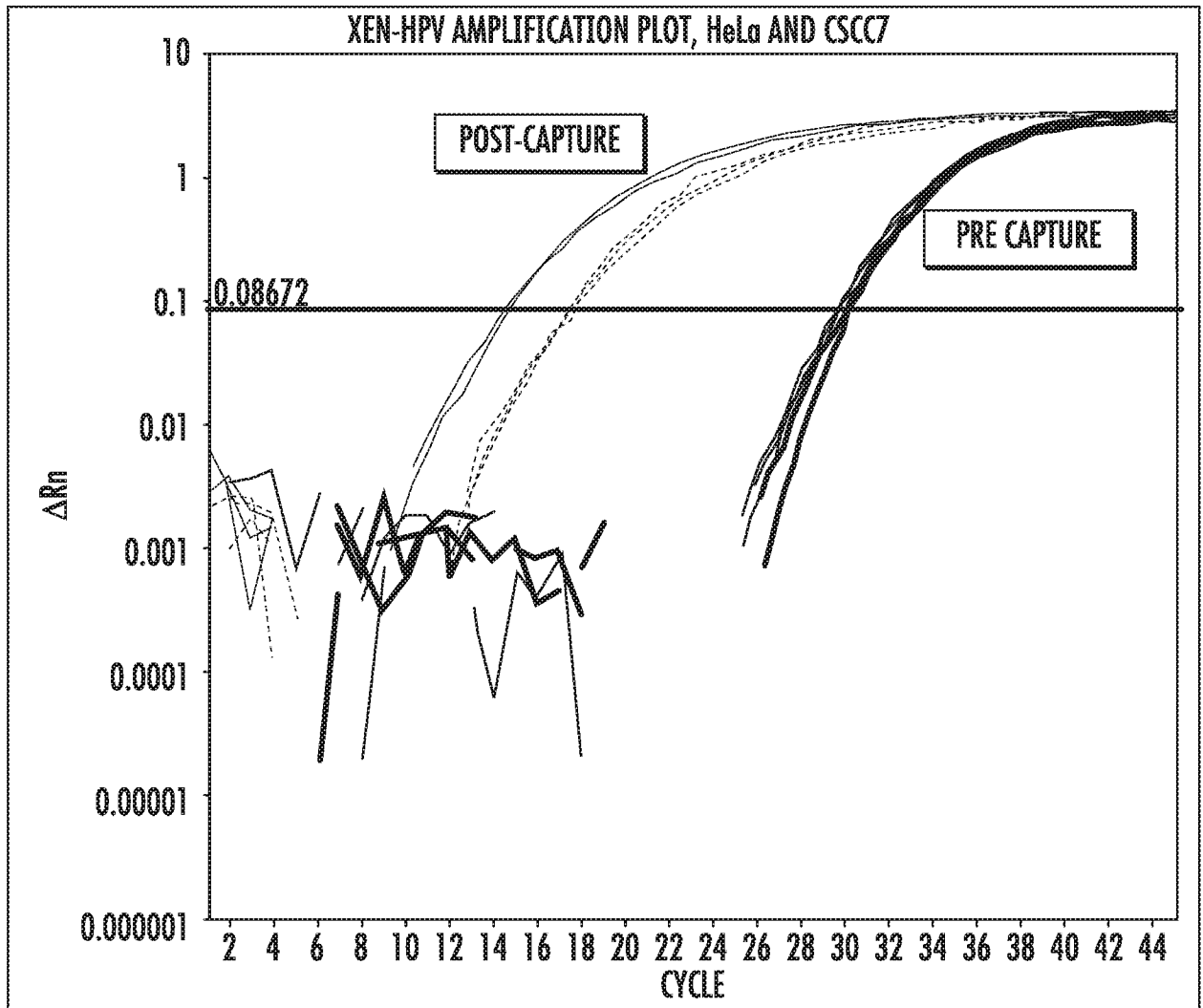


FIG. 8

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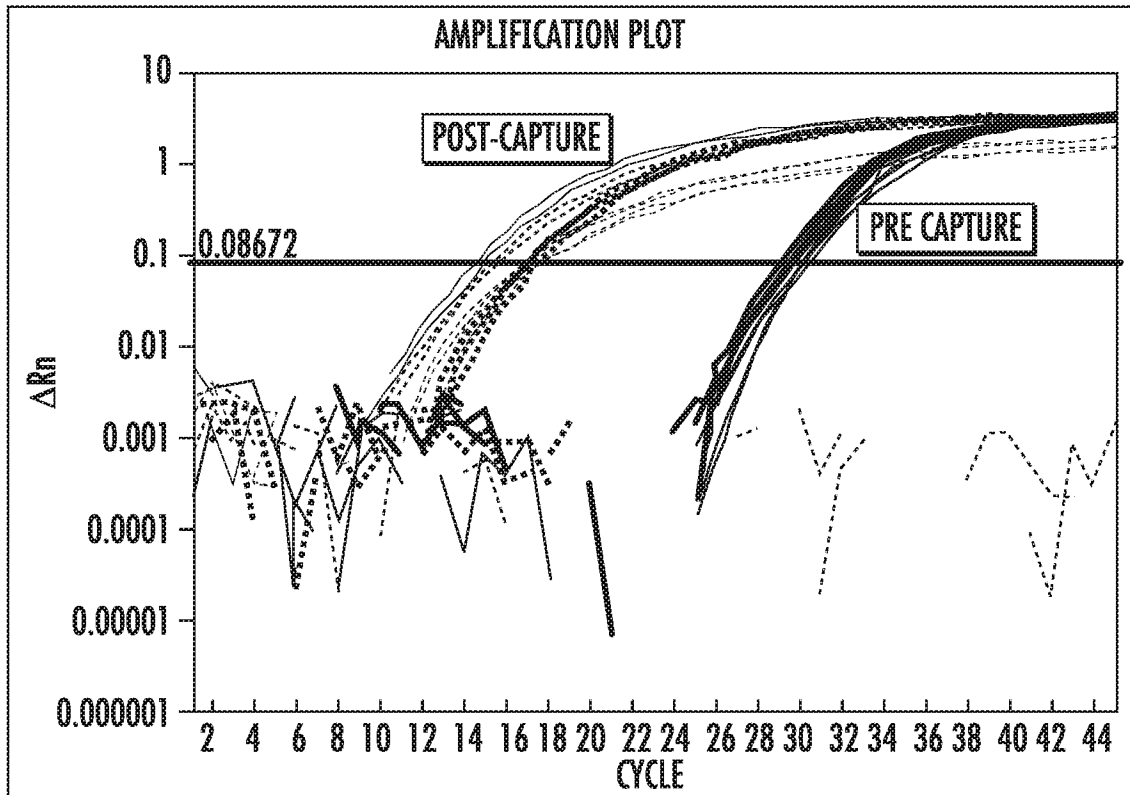


FIG. 9

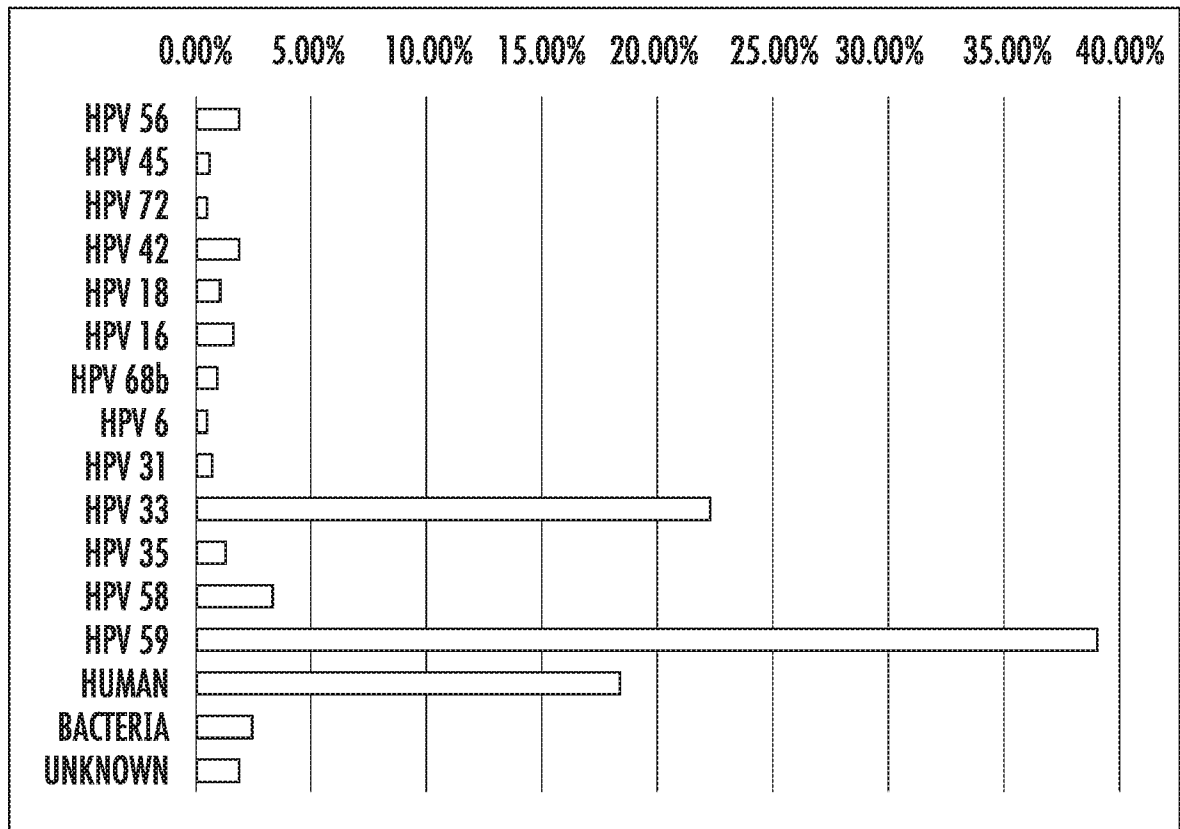


FIG. 10

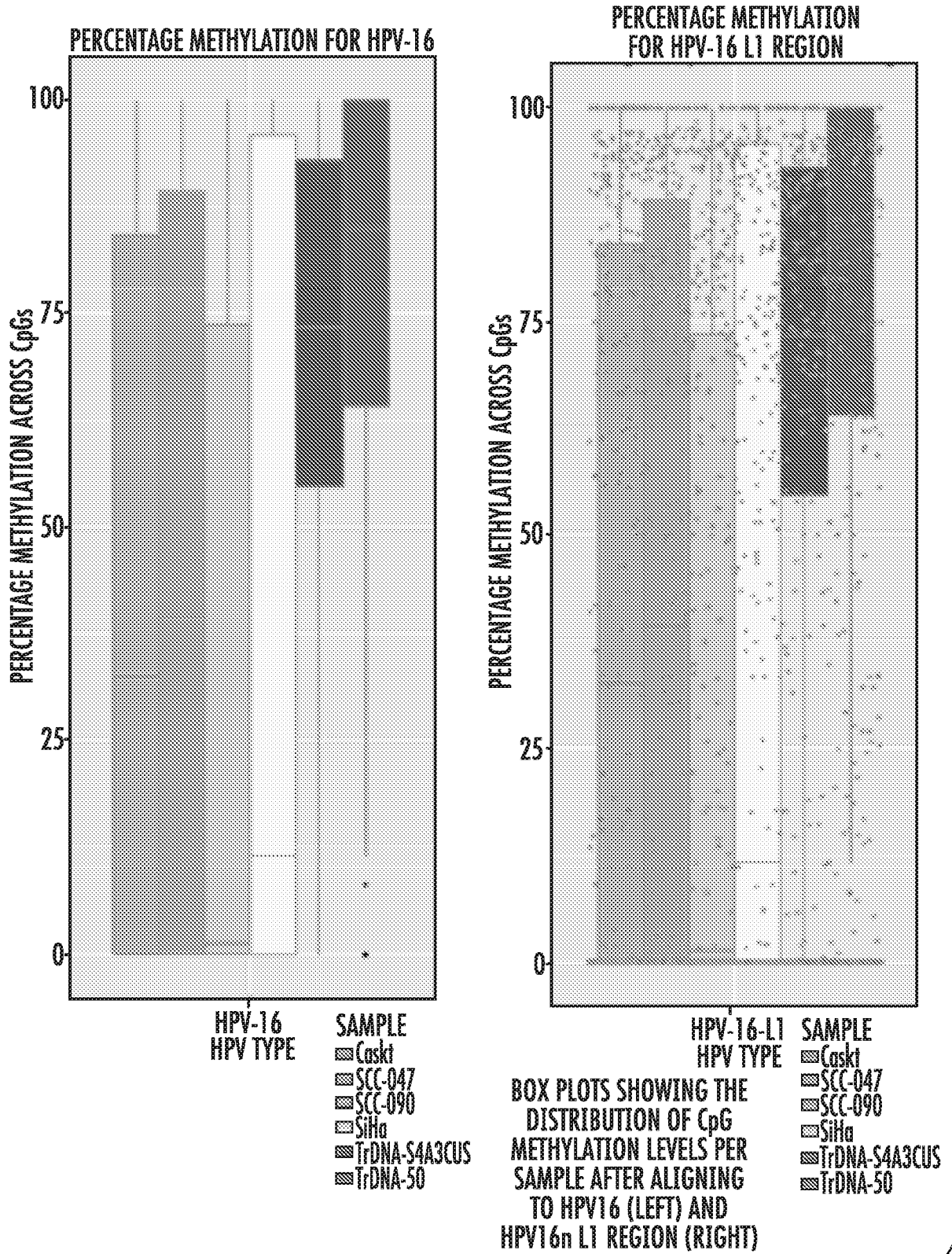


FIG. 11

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	PC	Tr-DNA
SAMPLE ID	ng/ul	ng/ul
301539	338.56	52.8
338542	887.38	31.46
10403	223.98	32.64
117139	206.46	39.35
381022	319.55	21.28
693696	369.83	37.17

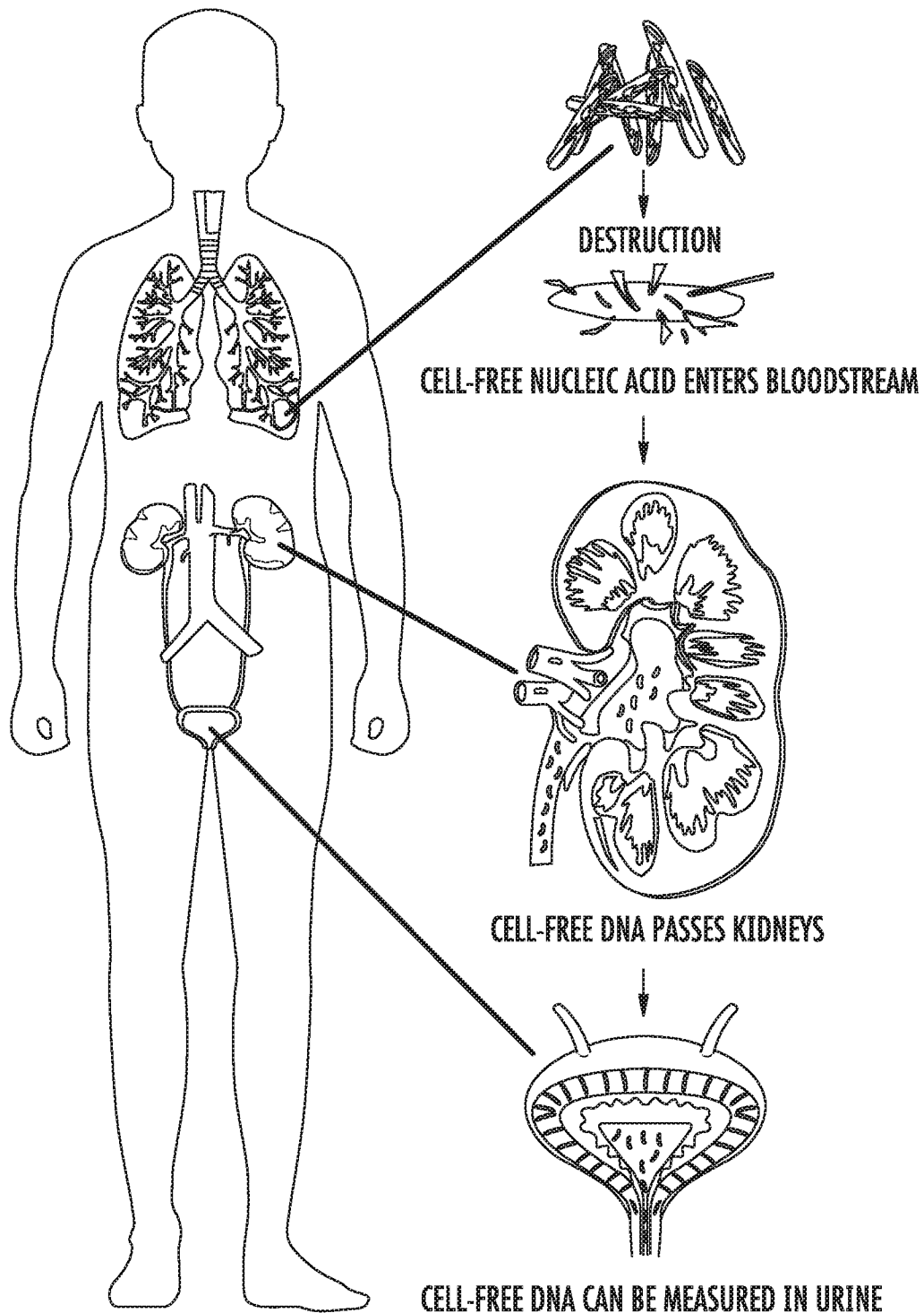


FIG. 13

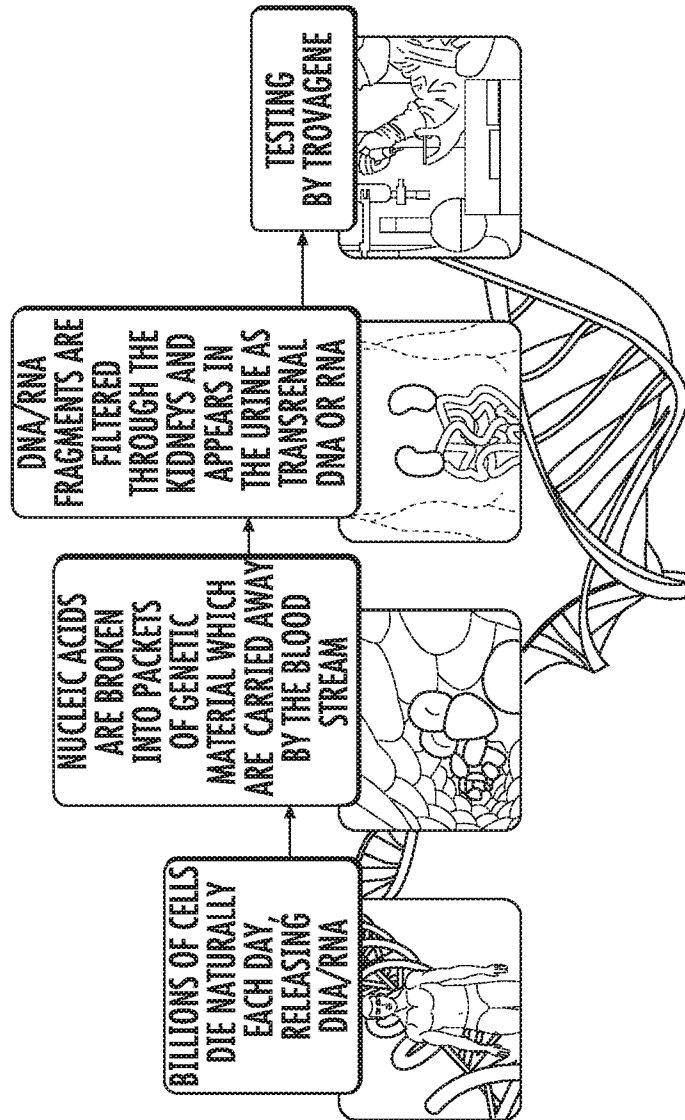


FIG. 13 (CONT.)

T ₁ DNA SAMPLE	LESION GRADE	READS TOTAL	MATCH TO HPV			MATCH TO HUMAN	MATCH TO BACTERIA	UNMAPPED
			HIGH (15) AND LOW RISK (6)	ALL	DOMINANT TYPES			
445	CIN3	25,998	25,618	25,838	16	154	6	0
455	CIN1	55,002	54,082	54,105	18	782	18	85
456	CIN1	45,982	35,069	35,422	59,33,58	8,319	1,110	850
481	CIN3	18,159	10,333	10,564	16, 18	6,838	659	79
504	CIN3	33,218	22,274	23,031	16, 18	6,933	2,752	451
513	CIN1	25,242	13,234	13,589	16,18,53	10,570	290	618
571	CIN3	26,472	18,714	19,080	16,18	7,259	108	18
AVERAGE		32,868	25,618	25,947	16,18	5,836	706	300

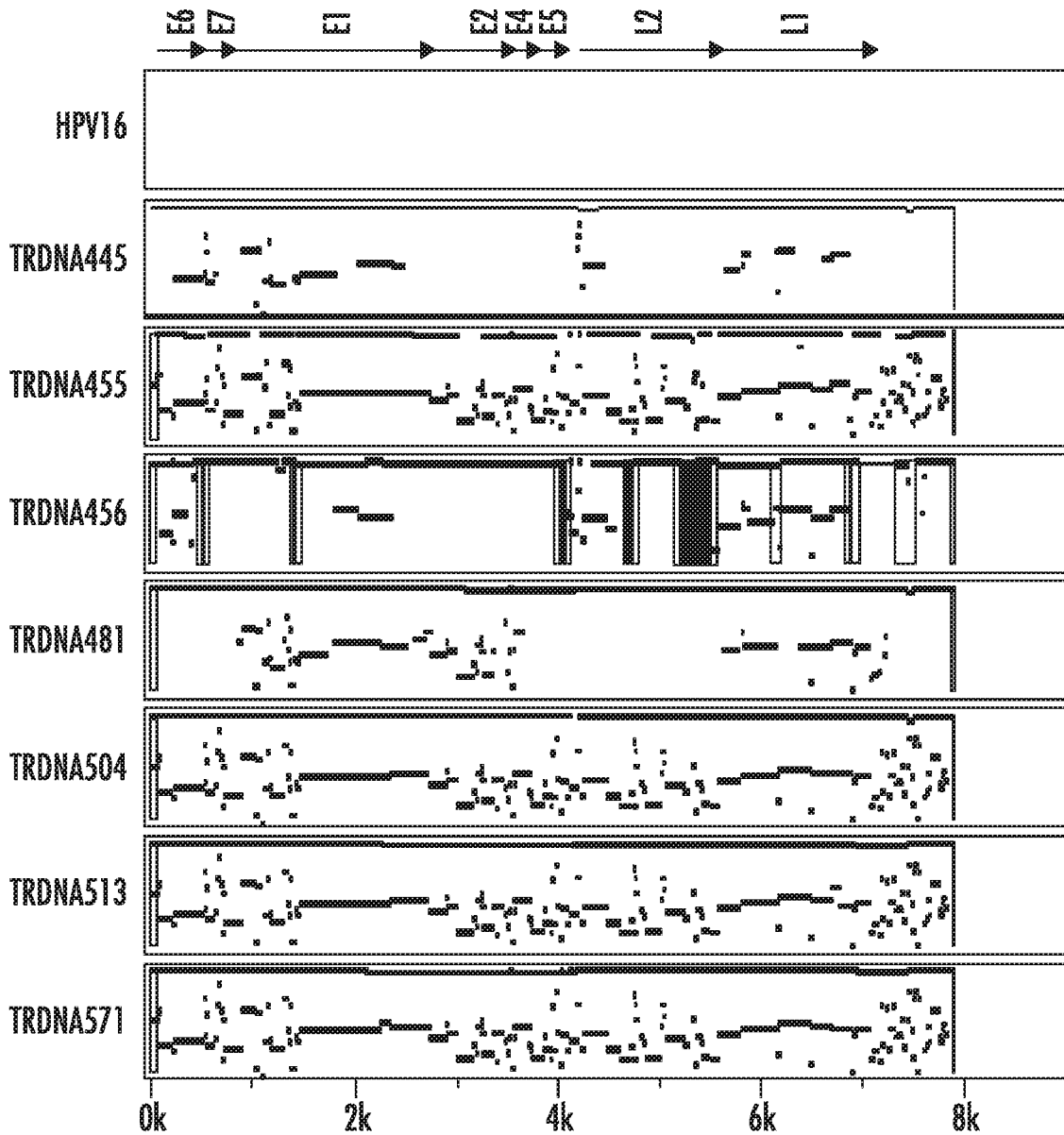


FIG. 14

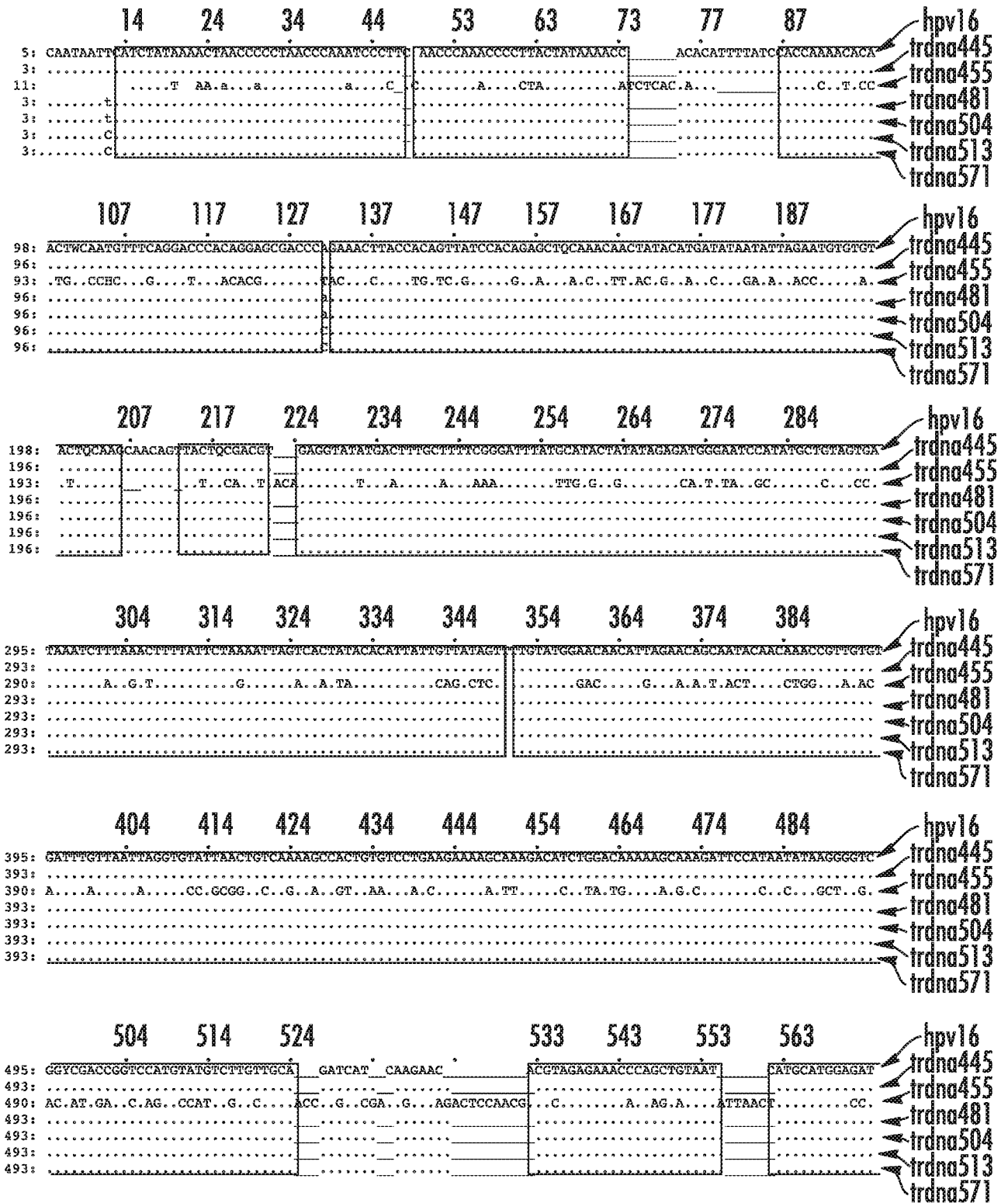


FIG. 15

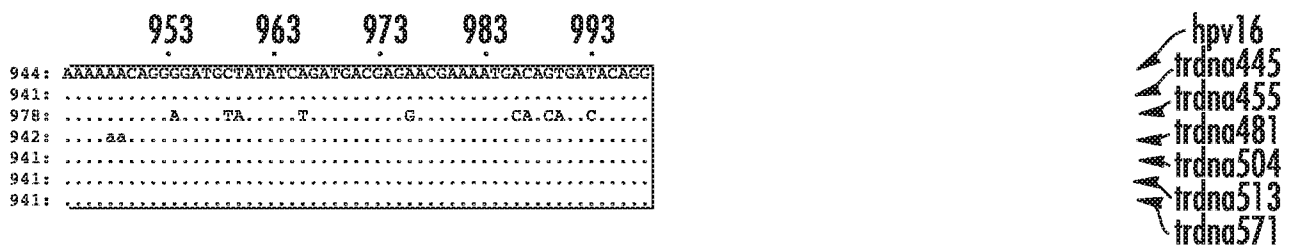
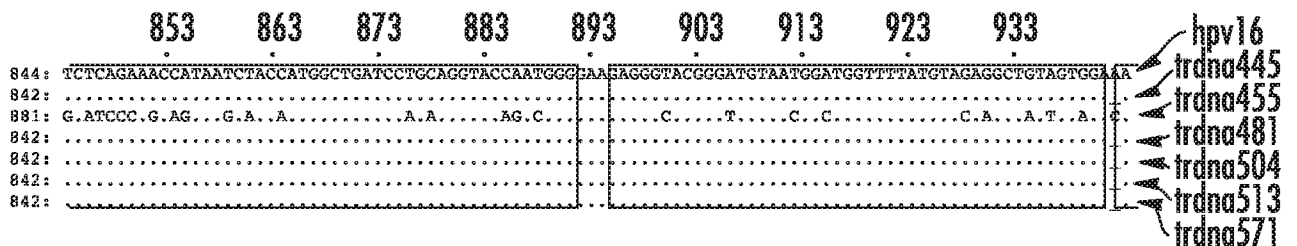
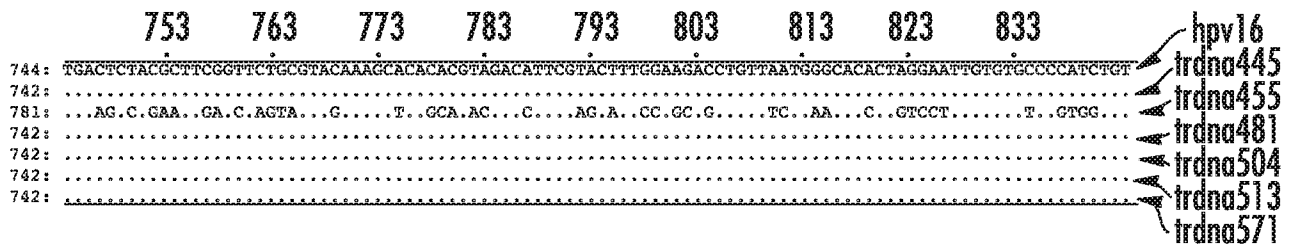
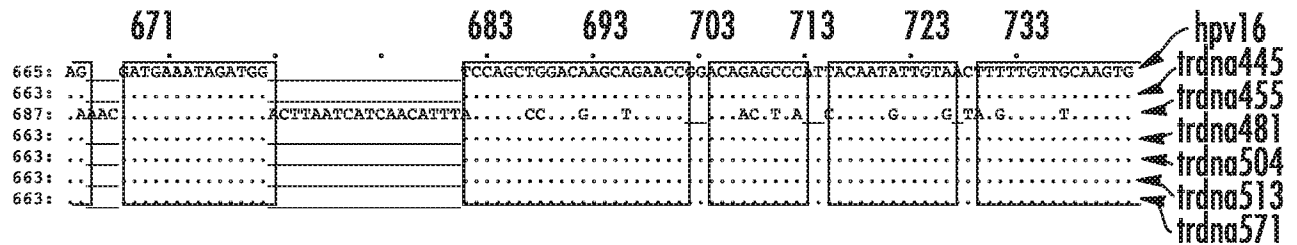
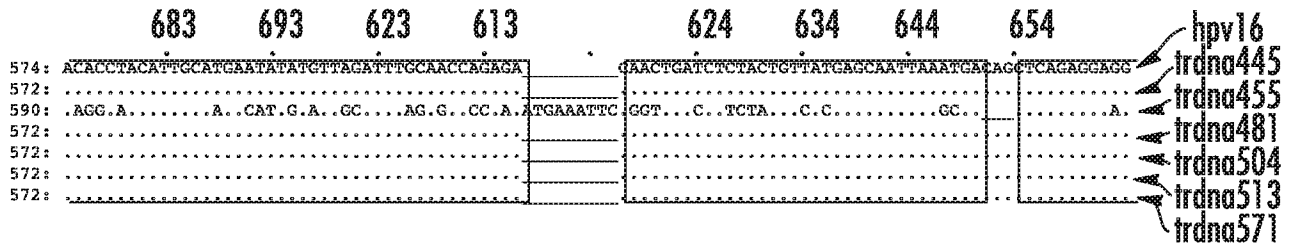


FIG. 15 (CONT.)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2016/044225

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.: 21-59
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

According to Rule 13.2 of the Regulations under the PCT the claimed inventions does not meet the requirement of unity of invention *a priori*, therefore independent claims 1, 4, 17, 18 form several groups of inventions, with are not linked as to form a single general inventive concept.

The special technical features of claims 1 and 4 are primers and/or probes specific for a group of genes comprising ZNF516, FKBP6, and INTS1 and HPV gene HPV16-L1.

The special technical features of independent claims 17 and 18 are a group of genes having methylated promoters.

Hence, claims comprise 2 groups of inventions, namely:

- 1 invention – claims 1-16
- 2 invention – claims 17-20

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2016/044225

A. CLASSIFICATION OF SUBJECT MATTER		<i>C12Q 1/68 (2006.01)</i> <i>C12N 15/11 (2006.01)</i>
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)		
C12Q 1/68, C12N 15/11		
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 practicable, search terms used)		
ESP@CENET, PAJ, PatSearch (RUPTO internal), RUPAT, USPTO, WIPO, PubMed		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	PRISCILLA BREBI et al. Genome-wide methylation profiling reveals Zinc finger protein 516 (ZNF516) and FK-506-binding protein 6 (FKBP6) promoters frequently methylated in cervical neoplasia, associated with HPV status and ethnicity in a Chilean population, Epigenetics, February 2014, Vol.9, no.2, pp.308-317	17-20 1-16
Y	ATTILA T LORINCZ et al. HPV16 L1 and L2 DNA methylation predicts high-grade cervical intraepithelial neoplasia in women with mildly abnormal cervical cytology, Int. J. Cancer, 2013, Vol.133, pp.637-645	1-16
Y	YAHYA I. ELSHIMALI et al. The Clinical Utilization of Circulating Cell Free DNA (CCFDNA) in Blood of Cancer Patients. Int. J. Mol. Sci., 2013, Vol.14, pp.18925-18958, especially p. 18926	1-3, 10
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents:		“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
“A”	document defining the general state of the art which is not considered to be of particular relevance	“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
“E”	earlier document but published on or after the international filing date	“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
“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)	“&” document member of the same patent family
“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	
Date of the actual completion of the international search		Date of mailing of the international search report
10 October 2016 (10.10.2016)		27 October 2016 (27.10.2016)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37		Authorized officer O. Fedonova Telephone No. 495 531 65 15