(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 September 2011 (09.09.2011)

PCT

(10) International Publication Number
WO 2011/109705 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2011/027181

(22) International Filing Date:
4 March 2011 (04.03.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/310,368 4 March 2010 (04.03.2010) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with information concerning one or more priority claims considered void (Rule 26bis.2(d))*

WO 2011/109705 A2

(54) Title: INTEGRATED ASSAY THAT COMBINES FLOW-CYTOMETRY AND MULTIPLEXED HPV GENOTYPE IDENTIFICATION

(57) Abstract: A two part assay is disclosed that enables collection of both protein biomarker phenotype and specific HPV genotype data from within a clinically derived population of cervical epithelial cells. Presence of multiple transformation-associated protein biomarkers acts as a gating criterion for cell sorting, followed by application of a PCR protocol sensitive enough to detect and identify individual HPV types from within the cells captured during sorting. The workflow has been optimized to work with cells conventionally fixed in PreservCyt (Cytyc), and it can be performed on residual cells remaining in a stored sample after a Pap test has been performed.

INTEGRATED ASSAY THAT COMBINES FLOW-CYTOMETRY AND MULTIPLEXED HPV GENOTYPE IDENTIFICATION

US Government Rights:

[0001] This disclosed assays and methods were invented with Government support under Contract No.: R21 CA125370, awarded by the National Institute of Health (NIH). The U.S. Government may have certain rights to this application.

BACKGROUND

Technical Field:

[0001] A two part assay is disclosed that enables collection of both protein biomarker phenotype and specific HPV genotype data from within a clinically derived population of cervical epithelial cells.

Description of the Related Art:

[0002] Cervical cancer is second to breast cancer as the most common form of malignancy in both incidence and mortality for women worldwide. The population-wide utilization of screening cervical cytology (Pap smear tests or "Pap tests") has been associated with a dramatic decrease in morbidity and mortality from cervical cancer in the United States and in other industrialized nations. Despite the success of Pap tests, the cytological diagnosis of cervical lesions is plagued by a persistent problem of low specificity for clinically significant high-grade lesions in patients with low-grade cytological abnormalities. As a result, over 4 million women each year receive a cytological diagnosis that requires further evaluation to rule out the possibility of high-grade dysplasia or cancer. In most cases, further evaluation

does not identify underlying high-grade lesions in patients with low-grade cytological abnormalities. Although a simple detection of high-risk types of human papillomavirus can play an important role for the triage of patients, the detection, by itself, has not been useful for several cytological diagnoses. Specifically, simple detection of high risk HPVs does not predict an underlying high grade lesion because of the long lag time (up to 10 years) between initial infection and development of disease. Further, some studies indicate that prognosis may vary even among separate high-risk HPV types, yet the current commercially available test for high-risk HPV does not specify individual HPV types.

[0003] Cytological diagnosis of premalignant lesions of the cervical mucosa includes premalignant lesions of the cervical mucosa, which are detected by cytological examination of the Papanicolaou preparation (Pap smear test). Cytological findings are classified by the Bethesda system as normal/benign reactive changes (Normal/BRC), squamous cell abnormalities, and glandular cell abnormalities. Squamous cell abnormalities include atypical squamous cells of undetermined significance (ASCUS), low grade squamous intraepithelial lesions (LSIL), encompassing evidence of human papillomavirus (HPV) infection and/or mild dysplasia, and high grade intraepithelial lesions (HSIL), including moderate dysplasia (CIN 2) and severe dysplasia/carcinoma in situ (CIN 3). In the revised Bethesda 2001 system, the general category of ASCUS was replaced with two new diagnostic categories: “atypical squamous cells - of undetermined significance” (ASC-US) and “atypical squamous cells - cannot exclude HSIL” (ASC-H). Cases that would previously be classified as ASCUS, favoring a reactive process, are included in the category of benign cellular changes in the new classification system. The classification of LSIL and HSIL remained

relatively unchanged from the previous Bethesda classification system but an additional subcategory of HSIL was created to report cases that have features that are suspicious for invasive carcinoma. Potentially premalignant lesions of the endocervical glandular mucosa can also be recognized in cervical cytological specimens. In the revised 2001 Bethesda system of cytological classification, glandular cell abnormalities that are less severe than adenocarcinoma are categorized as atypical glandular cells (AGC), either endocervical, endometrial, or "glandular cells" not otherwise specified (AGC NOS); atypical glandular cells, either endocervical cells or "glandular cells" favor neoplasia (AGC "favor neoplasia"), and endocervical adenocarcinoma in situ (AIS).

[0004] The Papanicolaou test is an effective screening tool for cancer. However, despite its success, the practice of diagnostic cytopathology is limited by poor specificity for underlying clinically significant lesions in cases with low-grade cytological abnormalities. Over 4 million cases are diagnosed as ASC-US, ASC-H, LSIL, or AGC in the United States each year, requiring further evaluation to identify the subset of patients that will have clinically significant high-grade lesions found on cervical biopsy (Table 1). In most cases, however, further evaluation does not identify high-grade squamous or glandular lesions in patients with low-grade cytological abnormalities. Patients with a cytological diagnosis of ASC (not otherwise specified) have a 5 to 17% of underlying CIN 2/3 on cervical biopsy and the diagnosis of ASC-H denotes a 24 to 94% chance of CIN 2/3 on colposcopic biopsy. In LSIL cases that were referred for colposcopic examination, high grade cervical dysplasia (CIN grade 2 or 3) was found in 25%, CIN 1 was found in 45%, but no dysplasia was found in over 25% of LSIL cases. The cytological diagnosis of AGC is also equivocal for the

presence of an underlying clinically significant lesion of the cervical mucosa. Cases with a diagnosis of AGC have been found to have a 9 to 41% risk of CIN2, CIN 3, or AIS but the majority of AGC cases do not have biopsy confirmation of clinically significant lesions of either the squamous or glandular mucosa.

Table 1. Clinically Equivocal Cytological Diagnostic Categories

Cytological Diagnosis	Total cases (Annual US population)*	No clinically significant lesion on colposcopy
ASC-US	> 2 million	1.66-1.9 million
ASC-H	0.20 million (estimated)	0.001-0.15 million
LSIL	1.65 million	1.24 million
AGC	0.31 million	0.18-0.25 million
Total	>4.16 million	2.66-3.54 million

[0005] The above statistics regarding performance of the Pap test of course do not even address the problem of lack of implementation in developing nations, where mortality rate per 100,000 women is 3-7 fold that of more industrialized areas of the world. Another issue with current practice is that clinicians are under pressure to read large numbers of specimens quickly. Cytotechnologists may have a screening rate of approximately 4-5 minutes per slide, or 100 slides in 8 hours. In other words, within 4 or 5 minutes, a cytotechnologist needs to visualize between 50,000 to 300,000 cells on a slide and to identify as few as 10 to 50 dysplastic cells in a positive specimen. Among all screened specimens, approximately 90% are negative. Therefore, most of the screener's time and energy is expended looking at healthy cells. Fatigue and monotony can reduce the acuity of the screener so that rare positive cells have a greater chance of being overlooked.

[0006] Cervical cancer detection at the molecular level includes detecting HPV oncogenes E6 and E7, and detecting $p16^{INK4a}$ overexpression. Specifically, human papillomavirus has been categorized into approximately 200 types that vary according to risk of cervical cancer onset. HPV types 16 and 18 are the most prevalent 'high-risk' types, associated with 70% of cervical cancers; remaining cases are nearly all positive for other, less common high-risk HPV types. The HPV genome contains two major oncogenes, E6 and E7. The E6 protein binds and induces the degradation of p53 via a ubiquitin-mediated process. E7 protein binds and destabilizes Rb and related proteins. Together, these effects promote cell-cycle progression and viral DNA replication in differentiated keratinocytes. In pre-cancerous cervical lesions and cervical carcinomas, human papillomavirus DNA integration into the host genome may result in disruption of the viral E2 open reading frame, resulting in unregulated overexpression of HPV oncogenes E6 and E7.

[0007] Cancers that have lost Rb gene function show overexpression of $p16^{INK4a}$, and a reciprocal correlation between pRb and $p16^{INK4a}$ has been established in several cancers. In most normal cells, $p16^{INK4a}$ expression is known to be low at both the mRNA and protein levels. During HPV infection, expression of HPV E7 protein causes inactivation of Rb by binding and directing it to be degraded via a ubiquitin-proteasome pathway. Accordingly, $p16^{INK4a}$ overexpression is observed in both established cervical cancer cell lines and human ectocervical cells immortalized by HPV types 16 and 18. In addition, upregulation of $p16^{INK4a}$ by HPV infection has been shown to vary between high- and low-risk HPV types, with strongest upregulation by high-risk type HPV16 relative to low-risk HPV6.

[0008] The $p16^{INK4a}$ protein has been used as an immunohistochemical and immunocytochemical marker to detect cervical cancer as follows. Some studies show very high levels of $p16^{INK4a}$ in almost 100% of high-grade cervical dysplasias and invasive cancers, whereas no $p16^{INK4a}$ -positive stain was found in normal cervical epithelia using the same antibodies. Further, an over-expression of $p16^{INK4a}$ and a decreased expression of Rb have been correlated with incidence of cervical dysplasia. Recent studies indicate that mcm5 may also be a marker for the presence of cervical intraepithelial neoplasia and carcinoma but can be expressed in low grade dysplastic lesions and in some normal proliferating squamous cells. It has been demonstrated that $p16^{INK4a}$ and mcm5 can be combined using immunological staining and flow cytometry to detect dual positive cells in a quantitative manner that tracks severity of cervical pathological state.

[0009] Cervical cancer detection at the molecular level (other protein biomarkers) has been demonstrated in several studies involving detection and quantification of cancer cell protein biomarkers by flow cytometry. Cancer biomarkers commonly detected by flow cytometry include ki67, PCNA, cyclin-D1 and cyclin-B1 and $p16^{INK4a}$. Most of these cancer biomarkers were primarily detected in lung, colon and breast cancer tissues. However, recent results show that increases in expression of $p16^{INK4a}$, mcm5 and cyclin D1 are also detected in cervical cancer samples. While this methodology is currently being developed for $p16^{INK4a}$, mcm5 and PCNA, the detection procedure is theoretically interchangeable for any biomarker with demonstrated expression in transformed cervical epithelial cells.

[0010] As the role of human papillomavirus in cervical cancer has unfolded, it has become clear that divergence of the virus into separate genetic lineages has resulted in entities that

vary in severity of host cell transformation and, ultimately, progress of disease. Five HPV genetic types are recognized as the most prevalent high-risk forms: 16, 18, 31, 33 and 45. Of these, it is widely recognized that type 16 appears in approximately half of all cervical cancers, yet HPV18 appears to consistently contribute to poorest prognoses even though it accounts for only ~20% of cervical cancers. An alternate way of detecting the dependence of disease severity upon HPV type shows that, when other cervical lesions are considered in addition to actual cases of cancer (ASCUS, LSIL, HSIL), it is clear that HPV types 16 and 18 vary in frequency among lesion classes in a manner reflecting the large relative risk attached to both. The combination of HPV genotyping data and pathological severity data among patients thus indicates that HPV type is information that has prognostic value for cases in which cell abnormality has begun. The problem up to this point is that an approximately 10 year latency between infection with high-risk HPV and onset of cancer prevents HPV type data by itself from being prognostically interpreted.

[0011] The Digene HPV HC2 test (Qiagen) uses a cocktail of RNA probes for 13 high risk HPV types, but a positive test result is ultimately detected by a generic antibody for DNA-RNA hybrids and does not indicate which HPV type is present. Furthermore, the HC2 test is limited by the absence of an internal control for specimen adequacy. Various PCR strategies have been used in research settings, but these usually involve nested PCR reactions that combine amplification with degenerate primers followed by secondary reactions with multiplexed, specific PCR primers.

SUMMARY OF THE DISCLOSURE

[0012] A combination of HPV type data with detection of transformation-associated protein biomarkers is disclosed for making the HPV genotype a more prognostically relevant tool. Specifically, an assay is disclosed that combines very high sensitivity and specificity that only requires a single amplification reaction after cell sorting isolates the target cells.

[0013] In one refinement, a two-part, integrated assay is disclosed that combines flow-cytometry and multiplexed HPV genotype identification. Protein biomarker phenotype and presence of specific HPV genotype is assessed for the same cell population within a clinical sample. Its purpose is to improve the overall accuracy and specificity of detection and characterization of incipient cervical disease. The assay is compatible with samples conventionally fixed with PreservCyt (Cytyc), and can thus be applied to residual cells initially collected as part of a normal cervical lavage. High speed cell sorting recovers cells positive for over-expression of multiple protein biomarkers reported to be collectively indicative of transformation. Once recovered, these same cells are checked for high-risk HPV genotypes by using a set of PCR primers carefully designed to operate in a multiplexed reaction with no need for pre-amplification by degenerate HPV primers. Finally, detection of individual HPV types can be performed by automated detection of amplified fragment size using the capillary electrophoresis platform of the GenomeLab GeXP (Beckman Coulter).

[0014] The following oligonucleotide sequences and their reverse compliments are also disclosed, wherein the sequences by convention are written in a 5' to 3' bond orientation:

Sequence 1 TGGACCGGTCGATGTATGTCTTGT

Sequence 2 TACGCACAACCGAAGCGTAGAGTC

Sequence 3 AGTGTGACTCTACGCTTCGGTTGT

Sequence 4 GTGTGCCCATTAACAGGTCTTCCA

Sequence 5 ACTATAGAGGCCAGTGCCATTCGT

Sequence 6 TCGTCGGGCTGGTAAATGTTGATG

Sequence 7 CATCAACATTTACCAGCCCGACGA

Sequence 8 AAACAGCTGCTGGAATGCTCGAAG

Sequence 9 AACATAGGAGGAAGGTGGACAGGA

Sequence 10 GTGTGCTCTGTACACACAAACGAAG

Sequence 11 GAGGACACAAGCCAACGTTAAAGG

Sequence 12 GGTTCGTAGGTCACTTGCTGTACT

Sequence 13 GACAGTACCGAGGGCAGTGTAATA

Sequence 14 TACTTGTGTTTCCCTACGTCTGCGA

Sequence 15 GCGTGTGTATTATGTGCCTACGCT

Sequence 16 TTACACTTGGGTCACAGGTCGG

Sequence 17 AGGTGACACTATAGAATATGGACCGGTCGATGTATGTCTTGT

Sequence 18 GTACGACTCACTATAGGGATACGCACAACCGAAGCGTAGAGTC

Sequence 19 AGGTGACACTATAGAATAAGTGTGACTCTACGCTTCGGTTGT

Sequence 20 GTACGACTCACTATAGGGAGTGTGCCCATTAACAGGTCTTCCA

Sequence 21 AGGTGACACTATAGAATAACTATAGAGGCCAGTGCCATTCGT

Sequence 22 GTACGACTCACTATAGGGATCGTCGGGCTGGTAAATGTTGATG

Sequence 23 AGGTGACACTATAGAATACATCAACATTTACCAGCCCGACGA

Sequence 24 GTACGACTCACTATAGGGAAAACAGCTGCTGGAATGCTCGAAG

Sequence 25 AGGTGACACTATAGAATAAACATAGGAGGAAGGTGGACAGGA

Sequence 26 GTACGACTCACTATAGGGAGTGTGCTCTGTACACACAAACGAAG

Sequence 27 AGGTGACACTATAGAATAGAGGACACAAGCCAACGTTAAAGG

Sequence 28 GTACGACTCACTATAGGGAGGTTCGTAGGTCACTTGCTGTACT

Sequence 29 AGGTGACACTATAGAATAGACAGTACCGAGGGCAGTGTAATA

Sequence 30 GTACGACTCACTATAGGGATACTTGTGTTTCCCTACGTCTGCGA

Sequence 31 AGGTGACACTATAGAATAGCGTGTGTATTATGTGCCTACGCT

Sequence 32 GTACGACTCACTATAGGGATTACACTTGGGTCACAGGTCGG

[0015] In another refinement, a sequential method is disclosed wherein cells exhibiting over-expression of one or more protein biomarkers are captured, via cell sorting of conventionally fixed and immunologically stained cervical cell populations, and then directly checked for human papillomavirus content. The method is categorically hierarchical and allows specific HPV genotype(s) to be attributed to a cell subpopulation that has already been characterized for protein biomarker phenotype. In this way cells are detected that individually exhibit combined risk factors for cervical disease, specifically indicating the presence of cell lineages that contain both protein biomarker indication of disease state and

presence of specific, high-risk HPV type(s). Without being bound to any particular theory, the cell sorting specifically acts as: 1) a method for rare event detection that proportionally quantifies occurrence of biomarker over-expression within a cervical cell population, and 2) a pre-filtering workflow that channels only abnormal cells toward the HPV assay. Within the disclosed method, gating criteria for cell sorting via flow cytometry may be set to isolate cells exhibiting over-expression of any two proteins among the set including, but not limited to, $p16^{INK4a}$, mcm5, PCNA, and any other protein biomarker associated with cervical epithelial cell transformation.

[0016] In a refinement, any or all of Sequences 17-32 are used to perform specific detection and identification of individual high-risk HPV types 16, 18, 31, 33, 45 and/or 52 by executing a single multiplexed PCR amplification directly from cells captured during cell sorting. Further, in this claim, individual HPV types are subsequently identified by resolving target amplicons via automated capillary electrophoresis.

[0017] In a refinement, any or all of Sequences 1-16, end-labeled with a fluorescent dye, are used to perform specific detection and identification of individual high-risk HPV types 16, 18, 31, 33, 45 and/or 52 by executing a single multiplexed PCR amplification from cells captured during cell sorting. In this claim, individual HPV types can subsequently be identified by resolving target amplicons via automated capillary electrophoresis. Alternately, in this claim, individual HPV types are represented by target amplicons of sufficiently different size so that detection can be performed with standard agarose gel electrophoresis.

[0018] In a refinement, sensitivity and specificity of HPV detection and identification is enhanced by using a thermocycling program optimized according to criteria normally used

for qPCR, in which there are only two cycle steps that respectively perform denaturation and annealing/extension during extremely short incubation times for ≥35 cycles.

[0019] A method is disclosed for constructing a clinical prognosis protocol based upon a matrix of protein biomarker phenotypes versus HPV types as determined via the methods described above. Protein biomarker phenotypes in this case specifically include both the particular protein biomarkers detected and their proportions within the cell population as determined by positive versus negative sorting events automatically counted during the cell sorting process. The combined protein and HPV type characterizations of individual clinical samples, obtained via the assays described above, are clustered with pathology data from case histories to produce a finely resolved association matrix for assay results versus predicted disease outcome.

[0020] Other advantages and features will be apparent from the following detailed description when read in conjunction with the attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] For a more complete understanding of the disclosed methods and apparatuses, reference should be made to the embodiments illustrated in greater detail in the accompanying drawings, wherein:

[0022] Figure 1 is a flow diagram for the cell sorting stage of a disclosed assay;

[0023] Figure 2 is a flow diagram for the PCR and capillary electrophoresis stage of a disclosed assay;

[0024] Figure 3 illustrates, graphically, clinically normal cervical cells characterized by signal intensity for immunofluorescent staining with antibodies for the human proteins $p16^{INK4a}$ and MCM5;

[0025] Figure 4 illustrates, graphically, low-grade squamous intraepithelial lesion cells characterized by signal intensity for immunofluorescent staining with antibodies for the human proteins $p16^{INK4a}$ and MCM5;

[0026] Figure 5 illustrates, graphically, high-grade squamous intraepithelial lesion cells characterized by signal intensity for immunofluorescent staining with antibodies for the human proteins $p16^{INK4a}$ and MCM5;

[0027] Figure 6 illustrates, illustrates, graphically, HeLa cells characterized by signal intensity for immunofluorescent staining with antibodies for the human proteins $p16^{INK4a}$ and MCM5;

[0028] Figure 7 illustrates, graphically, percent signal type by sample class across samples illustrated in Figures 3-6;

[0029] Figure 8 shows test results that demonstrate high sensitivity and specificity for the disclosed detection of HPV from pre-sorted cells; and

[0030] Figure 9 shows test results that demonstrate proof of concept for detection of fluorescence-tagged HPV amplicons, from multiplex PCR with fluorescence-tagged primers, using an automated capillary electrophoresis platform.

[0031] It should be understood that the drawings are not necessarily to scale and that the disclosed embodiments are sometimes illustrated diagrammatically and in partial views. In certain instances, details which are not necessary for an understanding of the disclosed methods and apparatuses or which render other details difficult to perceive may have been omitted. It should be understood, of course, that this disclosure is not limited to the particular embodiments illustrated herein.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

[0032] A two part assay is disclosed that enables collection of both protein biomarker phenotype and specific HPV genotype data from within a clinically derived population of cervical epithelial cells. Data is collected hierarchically. Presence of multiple transformation-associated protein biomarkers acts as a gating criterion for cell sorting, followed by application of a PCR protocol sensitive enough to detect and identify individual HPV types from within the cells captured during sorting. The workflow has been optimized to work with cells conventionally fixed in PreservCyt (Cytyc), and it can be performed on residual cells remaining in a stored sample after a Pap test has been performed.

[0033] Protein biomarker data is quantified proportionally within a cell population by counting positive versus negative sorting events. Thus biomarker data for a clinical sample is not a single value representing an overall staining intensity, but is instead a value reflecting the proportion of the cell population in which individual cells surpass an intensity threshold for each of two or more biomarkers. The gating criterion for a positive sorting event can be set as a combination of desired signal intensities for the protein biomarkers being used.

Biomarkers are detected through conventional immunological staining of fixed cells with fluorescently labeled antibodies, and can include, but are not limited to, proteins of reported association with transformation of cervical epithelial cells, such as $p16^{INK4a}$ and mcm5, and other proteins associated with progression of other malignancies, such as PCNA, caPCNA, mcm2, etc. Cells that pass the gating criterion are sorted directly into 0.2ml PCR wells (either as individual tubes or within 96 well PCR plates).

[0034] HPV detection and identification is performed by using a multiplexed PCR, using sorted cells directly as template with no further sample preparation. This reaction uses primer pairs that have been carefully designed according to three general criteria.

[0035] Primer pairs were designed to avoid covering annealing sites with positions known to be polymorphic among isolates of an HPV type. For each HPV type, all available isolates reported to GenBank, that included sequence for genes E6 and/or E7, were used to produce separate sequence alignments for each of those two genes. Alignments were assembled using sequence alignment tools such as ClustalX in order to align the open reading frames after virtual translation to amino acid sequence. Gaps were inserted manually for maximum preservation of homologous alignment of amino acid positions, after which gaps were then inserted into DNA versions of aligned sequences. Sequence positions that exhibited polymorphism within an HPV type were noted and tagged as "excluded regions" when the contiguous E6 - E7 query sequence was submitted to primer design tools such as PrimerQuest^{SM} (Integrated DNA Technologies) for primer design. In cases where potential primer sites with optimal kinetic properties were limited in number, primers were allowed to cover one polymorphic site only in the 5' half of the primer sequence. In this way, 3' end

stability should be preserved and the effect on overall sensitivity should be negligible. Tables 4A and 4B list the GenBank accession numbers for all HPV sequences used to produce within-type sequence alignments for genes E6 and E7. Finally, for a single HPV type, the query sequence submitted to PrimerQuest^{SM} consisted of a contiguous fragment covering the viral genome from the 5' end of E6 to the 3' end of E7. For each HPV type, this fragment was taken from the reference whole genome sequence submitted for that type. For HPV52, E7 was excluded from the design query because no isolates were available for detecting polymorphism. GenBank accession numbers for the whole genome reference sequences used for each type appear as the top accession number listed in each column in Tables 4A and 4B. PrimerQuest^{SM} was instructed to return 50 potential primer pairs for each query so that a final multiplex could be assembled by picking primer pairs that maximized sequence divergence among HPV types at annealing sites.

[0036] All primers were designed to be as similar as possible with regard to kinetic properties. T_m values were constrained within a range covering four degrees Celsius, with a difference no greater than two degrees allowed between any two primers of a matched pair. Primer length ranges from 22 to 25 nucleotides, with 13 of 16 all being 24 nucleotides in length. (Sequences 17 - 32 are just Sequences 1 - 16 with an 18-mer and 19-mer universal priming sequence attached - see description of the preferred detection embodiment of the invention below.) GC% ranges from 48% to 54%. 3' end stability scores range from approximately 3 to 7, according to the algorithm used by PrimerQuest^{SM}. If Sequences 1 - 16 are used, all target amplicons are less than 300bp. For Sequences 17 - 32, all target amplicons are less than 350bp. When combined with a high performance recombinant DNA

polymerase such as Platinum PFX (Invitrogen), allows for the use of stringent thermocycling parameters that mimic programs used for qPCR. This second design aspect simultaneously increases sensitivity and specificity, and contributes to the successful use of a single PCR to detect and identify individual HPV types in a multiplexed context.

[0037] Finally, a multiple sequence alignment of the contiguous E6 and E7 open reading frames was assembled for HPV types 16, 18, 31, 33, 45 and 52. The sequences were again taken from the reference whole genome sequences submitted to Genbank for each HPV type. Alignment was done by first translating the reading frames in GeneDoc 2.6.002 and then assembling alignments of the amino acid sequences in ClustalX. In this way all gaps are introduced in frame, and evolutionarily homologous positions are more likely to be aligned. Nucleotide sequences were then aligned manually to match the amino acid alignments by using the gap insertion and sliding functions in GeneDoc. Primer pairs for each HPV type were chosen by comparing annealing sites with homologous regions among all six aligned sequences. Primer pairs that covered annealing sites most divergent from other HPV types were chosen with specific attention to sequence divergence near the 3' end of each primer. Simultaneously, primer pairs were chosen so that the target amplicons for all HPV types differ from each other by at least 7bp. Table 2 lists Sequences 1-16 with their actual primer names and salient characteristics. Table 3 simply lists Sequences 17-32 with their primer names and target amplicon lengths.

[0038] Final detection of amplicons from the multiplexed PCR is performed by using an automated parallel capillary electrophoresis platform such as the GenomeLab GeXP Genetic Analysis System (Beckman Coulter). The PCR master mix contains two fluorescent dye-

labeled oligonucleotides that act as universal primers. The sequences of these oligonucleotides are complementary to, respectively, the 18 nucleotide sequence common to the 5' end of Sequences 17, 19, 21, 23, 25, 27, 29 and 31, and the 19 nucleotide sequence common to the 5' end of Sequences 18, 20, 22, 24, 26, 28, 30 and 32. Use of Sequences 17 - 32 in conjunction with the PCR master mix of the GenomeLab GeXP Start Kit allows for a kinetic turnover in which the HPV type-specific primers dominate target amplification for approximately the first three cycles, followed by a shift to dominance of priming by the dye-labeled universal primers. Detection and analysis is then performed using standard fragment analysis with the GenomeLab DNA Size Standard Kit - 400.

[0039] Sequences 1-16, directly end-labeled with a fluorescent dye, may be used to perform the multiplex PCR followed by amplicon detection via an automated parallel capillary electrophoresis platform such as the GenomeLab GeXP Genetic Analysis System (Beckman Coulter). This alternate method does not require use of the universal primers included in the PCR master mix of the GenomeLab GeXP Start Kit. Amplicons may alternately be detected by standard fragment size analysis via agarose gel electrophoresis. Gel resolution is enhanced by incorporating 50% Synergel (Diversified Biotech) with conventional molecular biology grade agarose. Assay performance is optimized for execution with Platinum PFX DNA polymerase (Invitrogen), following the thermocycling protocol listed below:

Step 1	95°C for 2 minutes initial denaturation/cell lysis
Step 2	95°C for 15 seconds
Step 3	62°C for 5 seconds

Step 4	72°C for 5 seconds
Step 5	GOTO Step 2 39X
Step 6	4°C hold

Table 2. HPV targeted primers without universal priming sequence

		Start	Tm	ES	PP	PS
HPV16E6E7F	TGGACCGGTCGATGTATGTCTTGT	415	60.0°C	5.17	5.12	272bp
HPV16E6E7R	TACGCACAACCGAAGCGTAGAGTC	686	60.9°C	5.32		
HPV18E6E7F	ACTATAGAGGCCAGTGCCATTCGT	398	60.0°C	5.91	0.42	247bp
HPV18E6E7R	TCGTCGGGCTGGTAAATGTTGATG	644	59.6°C	5.08		
HPV31E6E7F	AACATAGGAGGAAGGTGGACAGGA	379	59.4°C	5.87	5.2	291bp
HPV31E6E7R	GTGTGCTCTGTACACACAAACGAAG	669	58.4°C	5.75		
HPV33E6E7F	GAGGACACAAGCCAACGTTAAAGG	469	58.5°C	5.12	2.89	230bp
HPV33E6E7R	GGTTCGTAGGTCACTTGCTGTACT	698	58.6°C	4.74		
HPV45E6E7F	GACAGTACCGAGGGCAGTGTAATA	395	58.1°C	3.04	4.97	80bp
HPV45E6E7R	TACTTGTGTTTCCCTACGTCTGCGA	474	59.9°C	7.16		
HPV52E6E7F	GCGTGTGTATTATGTGCCTACGCT	182	59.5°C	7.13	7.82	266bp
HPV52E6E7R	TTACACTTGGGTCACAGGTCGG	447	59.2°C	6.75		

[0040] Start = 5' starting position within the reference sequence used as the target for primer design for each HPV type. Reverse primers are the reverse compliment of the annealing site within the reference sequence, so their starting position occurs at the 3' end of the annealing site relative to the reference sequence. ES = end stability. PP = pair penalty. PS = product size. ES and PP are as reported by PrimerQuest^{SM} (Integrated DNA Technologies).

Table 3. HPV targeted primers with added universal priming sequence

		Produc
HPV16E6E7FU	AGGTGACACTATAGAATATGGACCGGTCGATGTATGTCTTGT	30
HPV16E6E7RU	GTACGACTCACTATAGGGATACGCACAACCGAAGCGTAGAGTC	
HPV18E6E7FU	AGGTGACACTATAGAATAACTATAGAGGCCAGTGCCATTCGT	28
HPV18E6E7RU	GTACGACTCACTATAGGGATCGTCGGGCTGGTAAATGTTGATG	
HPV31E6E7FU	AGGTGACACTATAGAATAAACATAGGAGGAAGGTGGACAGGA	32
HPV31E6E7RU	GTACGACTCACTATAGGGAGTGTGCTCTGTACACACAAACGAAG	
HPV33E6E7FU	AGGTGACACTATAGAATAGAGGACACAAGCCAACGTTAAAGG	26
HPV33E6E7RU	GTACGACTCACTATAGGGAGGTTCGTAGGTCACTTGCTGTACT	
HPV45E6E7FU	AGGTGACACTATAGAATAGACAGTACCGAGGGCAGTGTAATA	11
HPV45E6E7RU	GTACGACTCACTATAGGGATACTTGTGTTTCCCTACGTCTGCGA	
HPV52E6E7FU	AGGTGACACTATAGAATAGCGTGTGTATTATGTGCCTACGCT	30
HPV52E6E7RU	GTACGACTCACTATAGGGATTACACTTGGGTCACAGGTCGG	

[0041] Start = 5' starting position within the reference sequence used as the target for primer design for each HPV type, as in Table 2. The additional universal priming sequence specifically allows the assay to incorporate automated detection of target amplicons by using a Genome Lab GeXP (Beckman Coulter).

Table 4A. Genbank accession numbers of isolates used for SNP exclusion within HPV types 16, 18 and 31.

HPV16		HPV18		HPV31	
E6	E7	E6	E7	E6	E7
NC_001526	NC_001526	NC_001357	NC_001357	J04353	J04353
EF122273	EU118173	EF422111	EF422144	FJ202003	EF422156
EF122274	AY686584	EF422110	EF422143	FJ202002	EF422155
EF122275	AY686583	EF422109	EF422142	EF422123	EF422154
EF122276	AY686582	EF202155	EF202143	EF422122	EF422153
EF122277	AY686581	EF202154	EF202144	EF422121	EF422152
EF122278	AY686580	EF202153	EF202145	EF422120	EF422151
EF122279	AY686579	EF202152	EF202146	EF422119	EF422150
EF122280	AF536180	EF202143	EF202147	EF566933	EF422149

EF122281	AF536179	EF202144	EF202148	EF422148
EF122282	AF534061	EF202145	EF202149	EF422147
EF122283	AF402678	EF202146	EF202150	EF422146
EF122284	AF472509	EF202147	EF202151	EF422145
EF122285	AF472508	EF661657	EF202152	
EF122286	AF125673	EF661656	EF202155	
EF122287	EF422141	EF202148	EF202154	
	EF422140	EF202149	EF202153	
	EF422139	EF202150	AY262282	
	EF422138	EF202150	X05015	
	EF422137	EF661655	U89349	
	EF422136	EF661654	Y18493	
	EF422135		Y18492	
	EF422134		Y18491	
	EF422133		A06324	
	EF422132		PPH18A	
	EF422131			
	EF422130			
	EF422129			
	EF422128			

[0042] In each case shown in Table 4A, the accession number listed at the top is the complete genome sequence for its respective HPV type. The query sequence for primer design in each case is the contiguous E6-E7 sequence copied from within the complete genome sequence. Separate alignments for genes E6 and E7 within a type were assembled using ClustalX. All variable sites observed among isolates within a type were subsequently excluded from primer designs where feasible. Where variable sites could not be avoided, primers were chosen that covered no more than one variable site near their 5' end.

Table 4B. Genbank accession numbers of isolates used for SNP exclusion within HPV types 33, 45 and 52

HPV33		HPV45		HPV52
E6	E7	E6	E7	E6
M12732	M12732	X74479	X74479	X74481

EF422127	A12360	EF202167	M38198	FJ002423
EF422126	A07020	EF202166	Y13218	EF566936
EF422125	EF422157	EF202165	EF202167	EF566935
EF422124	EF422158	EF202160	EF202166	EF566934
M12732		EF202159	EF202165	EF566923
A12360		EF202158	EF202164	EF566922
A07020		EF202157	EF202163	DQ057295
EF566921		EF202156	EF202162	DQ057294
EF566920		Y13218	EF202161	DQ057293
		M38198	EF202160	DQ057292
			EF202159	DQ057291
			EF202158	DQ057290
			EF202157	
			EF202156	
			AJ242956	

[0043] In each case shown in Table 4B, the accession number listed at the top is the complete genome sequence for its respective HPV type. Use of E6 and E7 sequences and performance of alignments is as in Table 4A. In the case of HPV52, only gene E6 is represented by multiple isolates, so primer design was restricted to E6.

[0044] Flow cytometry detection of dysplastic cells from clinical samples using immuno-staining for p16 and mcm5 is disclosed. Flow cytometry can detect dual positivity for overexpression of p16 and mcm5 in a manner that tracks cytomorphological classification of clinical samples. Cell populations from clinical samples classified as normal, LSIL and HSIL were analyzed, as well as HeLa cells. Figures 1-5 illustrate flow cytometry results for all cell populations. All cells were fixed in PreservCyt (Cytyc, 90%MeOH / 10%H2O). Cells were incubated with p16/FITC and mcm5/APC antibodies. Cell samples were gated on forward/side scatter diagram (upper left panel of Figures 2-5). Gated cells were analyzed for

levels of p16 and mcm5. The dual parameter diagram (upper right panel of Figures 2-5) shows percentages of cells negative or positive for either marker. Percentages of cells positive for each marker independently are shown in the lower left and right panel of Figures 2-5. Three independent experiments were performed for each tissue type. Figure 6 summarizes the percentage of cells displaying each signal type within each tissue type.

[0045] In FIG. 8, multiplexed PCR detection of HPV16, HPV18 and HPV45 with high sensitivity and specificity is demonstrated. Proof of principle is demonstrated with a 3-plex of PCR primer pairs. Primers were designed to produce amplicons of 272bp, 247bp and 80bp respectively for HPV16, HPV18 and HPV45. All amplicons fall within the contiguous region of the E6 and E7 open reading frames. The design strategy explicitly focused on achieving robust detection within each type while avoiding non-specific amplification. Within each HPV type, multiple sequence alignments of E6 and E7 were performed for isolates culled from the NCBI Nucleotide database in order to detect polymorphisms among clinical isolates. Tables 4A and 4B include the Genbank accession numbers for all isolates used to perform alignments for HPV16, HPV18 and HPV45. Primers were designed using PrimerQuest^{SM} (IDT). This design application allows exclusion of positions within the target sequence, ensuring that polymorphic sites do not affect annealing. Primer pairs were chosen so that GC%, T_m and 3' end stability values were as similar as possible, resulting in similar sensitivity of detection for separate HPV types within a multiplexed reaction. Finally, the primer sets were chosen to cover locations within E6-E7 that are highly divergent between any target HPV type and the others. Table 5 lists T_m and amplicon size for the primer pairs used in this 3-plex.

Table 5: Properties of PCR primer pairs targeted to HPV18, HPV16 and HPV45

	T_m	Target Size
HPV18E6E7F	60.0°C	247bp
HPV18E6E7R	59.6°C	
HPV16E6E7F	60.0°C	272bp
HPV16E6E7R	60.9°C	
HPV45E6E7F	58.1°C	80bp
HPV45E6E7R	59.9°C	

[0046] The specificity and sensitivity of the HPV16/18/45 3-plex were tested using cell lines HeLa, SiHa and MS751, which respectively contain integrated genomic copies of HPV18, HPV16 and HPV45. Cells were first fixed in 90% methanol, as would normally happen if a clinical sample were fixed in PreservCyt (Cytyc). Cells were incubated with p16/FITC and mcm5/APC antibodies and sorted as above. Bulk sorted cells were resuspended in 90% methanol. Suspensions were made with 20 cells per microliter of a single type and 20 of each cell type per microliter. Sorted cells were used directly as PCR template by placing 1µl aliquots of cell suspensions in 0.2ml PCR tubes and drying in a Speed Vac (Savant) with no heat. 25µl aliquots of PCR master mix were placed directly on top of dried cells. In parallel, as positive controls to rule out amplification artifacts from cell material, an identical set of amplifications was performed using genomic DNA purified from the same cell lines. Positive control reactions contained 10ng of one DNA type (lanes 12-17) or 10ng of each DNA type (lanes 18-19). Figure 7 demonstrates high specificity and specificity of HPV detection. First, when directed against ~20 cells of each cell line individually (lanes 4-9), the multiplex of three primer pairs only produces an amplicon of the correct target size for the HPV type expected in each case. Second, in all of those reactions,

no undesigned amplicons appear as a result of nonspecific amplification from human genomic material. Third, when all three cell lines are mixed, the 3-plex is able to detect all three HPV types with the same visible intensity, showing that amplification efficiencies of targets are not interfered with by the presence of multiple HPV types in a reaction. Reactions using purified cell line genomic DNA produce an identical set of amplicons and relative intensities, so other cell-derived materials are not producing amplification artifacts. Finally, these tests used a thermocycling program that ran for 40 cycles in order to see whether a large number of cycles would be permitted to increase sensitivity. The fact that these clear results occur even after 40 cycles indicates the robust specificity of the primer design.

[0047] In Figure 7: Lane 1, 25bp DNA stepladder (Promega); Lanes 2-3, no template control-- 25µl aliquots of PCR master mix with 1µl H_2O as mock template addition; Lanes 4-9 replicate pairs of the 3-plex primer set applied to each cell line individually (each reaction contains ~20 cells that have passed through the immunostaining and sorting process); Lanes 10-11-- two replicates of the 3-plex primer set applied to all three cell lines mixed together (~20 cells of each line); Lanes 12-19--purified genomic DNA from the three cell lines used to reproduce the same pattern of template types as lanes 4-11; Single cell type reactions (lanes 12-17) contained 10ng genomic DNA from each respective cell line, while lanes 18 and 19 show two replicates of amplification from a template mix with 10ng of each DNA type. Gel is 1.25% agarose, 1.25% Synergel (Diversified Biotech) in 1X TAE. Separation performed at 70V for approximately 30 minutes. Gel post-stained with ethidium bromide.

[0048] Proof of principle for detection of amplicons via the capillary electrophoresis platform is demonstrated by using chimeric versions of the same primer 3-plex to which the

universal primer complementary sequences have been added. These chimeric primers correspond to Sequences 17, 18, 21, 22, 29 and 30 in Claim 1. These primers were targeted at template consisting of a mix of purified DNA from HeLa, SiHa and MS751 cells, 10ng of each per 1µl of solution. Amplification was performed using the 5X PCR buffer included in the GenomeLab GeXP Start Kit (Beckman Coulter, Inc.), which includes forward and reverse universal primers complementary to the 5' ends of the chimeric primers. The reverse universal primer is 5' end-labeled with WellRED D4, a far-red fluorescent dye with an emission peak at 670nm. Three replicate reactions were performed for both the positive detection trial and a no template control condition in which water was added in place of template DNA. A three-phase thermal cycling program was used to exploit the three different annealing conditions that occur sequentially when using this PCR chemistry (see the left side of Figure 2 for an illustration of the three annealing events). The thermal cycling program is as follows:

	1	95°C 10 minutes
	2	94°C 15 seconds
	3	65°C 20 seconds
	4	72°C 30 seconds
	5	GOTO 2 2X
	6	94°C 15 seconds
	7	72°C 1 minute
	8	GOTO 6 4X
	9	94°C 30 seconds

10 55°C 30 seconds

11 70°C 1 minute

12 GOTO 9 34X

13 4°C hold

Following thermal cycling, samples for fragment analysis were produced by combining 0.5µl PCR reaction with 39µl GeXP Sample Loading Solution (Beckman Coulter, Inc.) and 0.5µl DNA Size Standard – 400 (Beckman Coulter, Inc.). Fragment separation samples were prepared for each replicate of both the positive detection trial and the no template control. Fragment separation and detection were performed using the method "Frag-3" within the device controller software for the GenomeLab GeXP Genetic Analysis System. Figure 9 illustrates successful detection of electropherogram peaks specific to predicted amplicon sizes for HPV16, 18 and 45. All three peaks are absent from the no-template control.

[0049] While only certain embodiments have been set forth, alternatives and modifications will be apparent from the above description to those skilled in the art. These and other alternatives are considered equivalents and within the spirit and scope of this disclosure.

Claims

1. A method for detecting high-risk HPV genotypes in a cervical cell sample, the method comprising:

collecting the sample from a cervical lavage or a Pap test;

fixing cells of the sample with fluorescently tagged antibodies targeted to multiple biomarkers;

separating cells positive for multiple biomarkers from cells positive for one or less biomarkers;

applying a polymerase chain reaction (PCR) master mix to the cells positive for multiple biomarkers, the master mix comprising a plurality of PCR primers, the PCR primers comprising oligonucleotide sequences that produce within-type sequences for at least one of genes E6 and E7 of at least one HPV type selected from the group consisting of 16, 18, 31, 33, 45 and 53;

executing a single multiplexed PCR amplification to produce a plurality of amplified end-label amplicons; and

identifying HPV types in the plurality of amplified end-label amplicons using electrophoresis.

2. The method of claim 1 wherein the oligonucleotide sequences are selected from the group consisting of:

Sequence 1	TGGACCGGTCGATGTATGTCTTGT
Sequence 2	TACGCACAACCGAAGCGTAGAGTC

Sequence 3 AGTGTGACTCTACGCTTCGGTTGT
Sequence 4 GTGTGCCCATTAACAGGTCTTCCA

Sequence 5 ACTATAGAGGCCAGTGCCATTCGT
Sequence 6 TCGTCGGGCTGGTAAATGTTGATG

Sequence 7 CATCAACATTTACCAGCCCGACGA
Sequence 8 AAACAGCTGCTGGAATGCTCGAAG

Sequence 9 AACATAGGAGGAAGGTGGACAGGA
Sequence 10 GTGTGCTCTGTACACACAAACGAAG

Sequence 11 GAGGACACAAGCCAACGTTAAAGG
Sequence 12 GGTTCGTAGGTCACTTGCTGTACT

Sequence 13 GACAGTACCGAGGGCAGTGTAATA
Sequence 14 TACTTGTGTTTCCCTACGTCTGCGA

Sequence 15 GCGTGTGTATTATGTGCCTACGCT
Sequence 16 TTACACTTGGGTCACAGGTCGG

Sequence 17 AGGTGACACTATAGAATATGGACCGGTCGATGTATGTCTTGT
Sequence 18 GTACGACTCACTATAGGGATACGCACAACCGAAGCGTAGAGTC

Sequence 19 AGGTGACACTATAGAATAAGTGTGACTCTACGCTTCGGTTGT
Sequence 20 GTACGACTCACTATAGGGAGTGTGCCCATTAACAGGTCTTCCA

Sequence 21 AGGTGACACTATAGAATAACTATAGAGGCCAGTGCCATTCGT
Sequence 22 GTACGACTCACTATAGGGATCGTCGGGCTGGTAAATGTTGATG

Sequence 23 AGGTGACACTATAGAATACATCAACATTTACCAGCCCGACGA
Sequence 24 GTACGACTCACTATAGGGAAAACAGCTGCTGGAATGCTCGAAG

Sequence 25 AGGTGACACTATAGAATAAACATAGGAGGAAGGTGGACAGGA
Sequence 26 GTACGACTCACTATAGGGAGTGTGCTCTGTACACACAAACGAAG

Sequence 27 AGGTGACACTATAGAATAGAGGACACAAGCCAACGTTAAAGG
Sequence 28 GTACGACTCACTATAGGGAGGTTCGTAGGTCACTTGCTGTACT

Sequence 29 AGGTGACACTATAGAATAGACAGTACCGAGGGCAGTGTAATA
Sequence 30 GTACGACTCACTATAGGGATACTTGTGTTTCCCTACGTCTGCGA

Sequence 31 AGGTGACACTATAGAATAGCGTGTGTATTATGTGCCTACGCT
Sequence 32 GTACGACTCACTATAGGGATTACACTTGGGTCACAGGTCGG

and the reverse compliments thereof.

3. The method of claim 1 wherein the oligonucleotide sequences are selected from the group consisting of:

Sequence 1	TGGACCGGTCGATGTATGTCTTGT
Sequence 2	TACGCACAACCGAAGCGTAGAGTC
Sequence 3	AGTGTGACTCTACGCTTCGGTTGT
Sequence 4	GTGTGCCCATTAACAGGTCTTCCA
Sequence 5	ACTATAGAGGCCAGTGCCATTCGT
Sequence 6	TCGTCGGGCTGGTAAATGTTGATG
Sequence 7	CATCAACATTTACCAGCCCGACGA
Sequence 8	AAACAGCTGCTGGAATGCTCGAAG
Sequence 9	AACATAGGAGGAAGGTGGACAGGA
Sequence 10	GTGTGCTCTGTACACACAAACGAAG
Sequence 11	GAGGACACAAGCCAACGTTAAAGG
Sequence 12	GGTTCGTAGGTCACTTGCTGTACT
Sequence 13	GACAGTACCGAGGGCAGTGTAATA
Sequence 14	TACTTGTGTTTCCCTACGTCTGCGA
Sequence 15	GCGTGTGTATTATGTGCCTACGCT
Sequence 16	TTACACTTGGGTCACAGGTCGG

and the reverse compliments thereof.

4. The method of claim 1 wherein the oligonucleotide sequences are selected from the group consisting of:

Sequence 17	AGGTGACACTATAGAATATGGACCGGTCGATGTATGTCTTGT
Sequence 18	GTACGACTCACTATAGGGATACGCACAACCGAAGCGTAGAGTC
Sequence 19	AGGTGACACTATAGAATAAGTGTGACTCTACGCTTCGGTTGT
Sequence 20	GTACGACTCACTATAGGGAGTGTGCCCATTAACAGGTCTTCCA

Sequence 21 AGGTGACACTATAGAATAACTATAGAGGCCAGTGCCATTCGT
Sequence 22 GTACGACTCACTATAGGGATCGTCGGGCTGGTAAATGTTGATG

Sequence 23 AGGTGACACTATAGAATACATCAACATTTACCAGCCCGACGA
Sequence 24 GTACGACTCACTATAGGGAAAACAGCTGCTGGAATGCTCGAAG

Sequence 25 AGGTGACACTATAGAATAAACATAGGAGGAAGGTGGACAGGA
Sequence 26 GTACGACTCACTATAGGGAGTGTGCTCTGTACACACAAACGAAG

Sequence 27 AGGTGACACTATAGAATAGAGGACACAAGCCAACGTTAAAGG
Sequence 28 GTACGACTCACTATAGGGAGGTTCGTAGGTCACTTGCTGTACT

Sequence 29 AGGTGACACTATAGAATAGACAGTACCGAGGGCAGTGTAATA
Sequence 30 GTACGACTCACTATAGGGATACTTGTGTTTCCCTACGTCTGCGA

Sequence 31 AGGTGACACTATAGAATAGCGTGTGTATTATGTGCCTACGCT
Sequence 32 GTACGACTCACTATAGGGATTACACTTGGGTCACAGGTCGG

and the reverse compliments thereof.

5. The method of claim 1 wherein the separating of cells positive for multiple biomarkers comprises separating cells exhibiting over expression of any two proteins selected from the group consisting of $p16^{INK4a}$, mcm5, PCNA, and any other protein biomarker associated with cervical epithelial cell transformation.

6. The method of claim 3 wherein the electrophoresis is gel electrophoresis.

7. The method of claim 4 wherein the electrophoresis is capillary electrophoresis.

8. The method of claim 1 further comprising thermocycling the amplified end-label amplicons, wherein the thermocycling comprises a plurality of repeated cycles and each cycle comprises a denaturation step and an annealing/extension step.

9. The method of claim 8 wherein the thermocycling comprises less than about 35 cycles.

10. The method of claim 1 wherein the executing a single multiplexed PCR amplification to produce a plurality of amplified end-label amplicons is carried out without a pre-amplification by degenerate HPV primers.

11. The method of claim 1 wherein the fluorescently tagged antibodies, used during cell sorting, are directed toward two or more protein biomarkers known to have functional relationships with transformation, tumor initiation, tumor progression, or tumor invasion, such as, but not limited to, EGFR1, VEGF, HIF-1α, IGFBP-3, P-cadherin, MCM7, $p16^{INK4a}$ or MCM5.

12. An oligonucleotide sequence that produce within-type sequences for at least one of genes E6 and E7 of at least one HPV type selected from the group consisting of 16, 18, 31, 33, 45 and 53, the oligonucleotide sequence selected from the group consisting of:

Sequence 1 TGGACCGGTCGATGTATGTCTTGT
Sequence 2 TACGCACAACCGAAGCGTAGAGTC

Sequence 3 AGTGTGACTCTACGCTTCGGTTGT

Sequence 4	GTGTGCCCATTAACAGGTCTTCCA
Sequence 5	ACTATAGAGGCCAGTGCCATTCGT
Sequence 6	TCGTCGGGCTGGTAAATGTTGATG
Sequence 7	CATCAACATTTACCAGCCCGACGA
Sequence 8	AAACAGCTGCTGGAATGCTCGAAG
Sequence 9	AACATAGGAGGAAGGTGGACAGGA
Sequence 10	GTGTGCTCTGTACACACAAACGAAG
Sequence 11	GAGGACACAAGCCAACGTTAAAGG
Sequence 12	GGTTCGTAGGTCACTTGCTGTACT
Sequence 13	GACAGTACCGAGGGCAGTGTAATA
Sequence 14	TACTTGTGTTTCCCTACGTCTGCGA
Sequence 15	GCGTGTGTATTATGTGCCTACGCT
Sequence 16	TTACACTTGGGTCACAGGTCGG
Sequence 17	AGGTGACACTATAGAATATGGACCGGTCGATGTATGTCTTGT
Sequence 18	GTACGACTCACTATAGGGATACGCACAACCGAAGCGTAGAGTC
Sequence 19	AGGTGACACTATAGAATAAGTGTGACTCTACGCTTCGGTTGT
Sequence 20	GTACGACTCACTATAGGGAGTGTGCCCATTAACAGGTCTTCCA
Sequence 21	AGGTGACACTATAGAATAACTATAGAGGCCAGTGCCATTCGT
Sequence 22	GTACGACTCACTATAGGGATCGTCGGGCTGGTAAATGTTGATG
Sequence 23	AGGTGACACTATAGAATACATCAACATTTACCAGCCCGACGA
Sequence 24	GTACGACTCACTATAGGGAAAACAGCTGCTGGAATGCTCGAAG
Sequence 25	AGGTGACACTATAGAATAAACATAGGAGGAAGGTGGACAGGA
Sequence 26	GTACGACTCACTATAGGGAGTGTGCTCTGTACACACAAACGAAG
Sequence 27	AGGTGACACTATAGAATAGAGGACACAAGCCAACGTTAAAGG
Sequence 28	GTACGACTCACTATAGGGAGGTTCGTAGGTCACTTGCTGTACT
Sequence 29	AGGTGACACTATAGAATAGACAGTACCGAGGGCAGTGTAATA
Sequence 30	GTACGACTCACTATAGGGATACTTGTGTTTCCCTACGTCTGCGA
Sequence 31	AGGTGACACTATAGAATAGCGTGTGTATTATGTGCCTACGCT
Sequence 32	GTACGACTCACTATAGGGATTACACTTGGGTCACAGGTCGG

and the reverse compliments thereof.

12. A PCR primer comprising an oligonucleotide sequence of claim 11.

13. A PCR master mix comprising at least one PCR primer of claim 12.

FIG. 1

Workflow for cell sorting and staging of PCR reactions

1. Cells fixed and incubated with fluorescently tagged antibodies targeted to protein biomarkers.

2. Cells positive for multiple markers sorted, sorting events counted for quantitative marker phenotype.

3. PCR master mix applied directly to sorted cells. Primers target multiple high-risk HPV types.

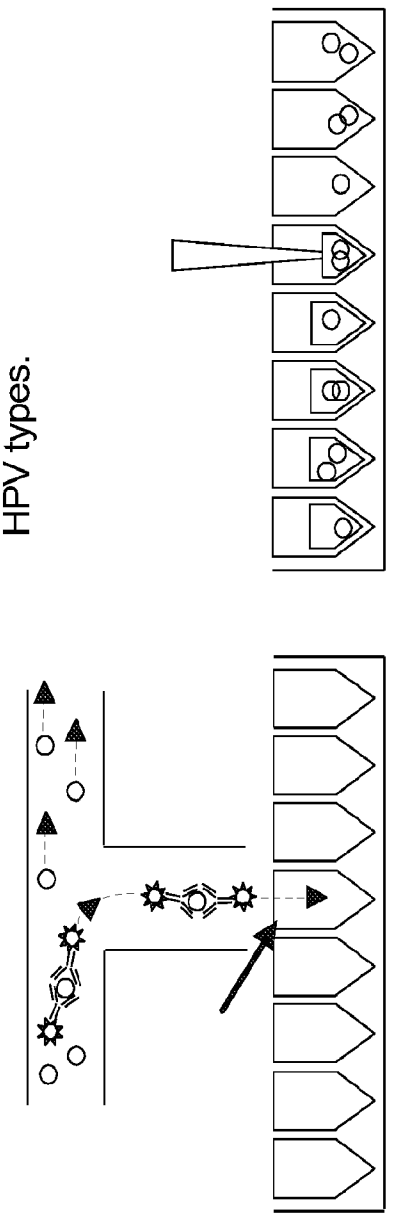
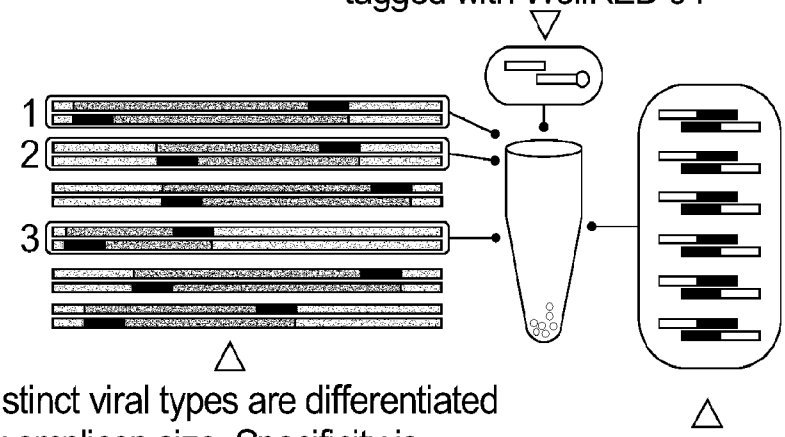


FIG. 2

Labeled universal primer strategy

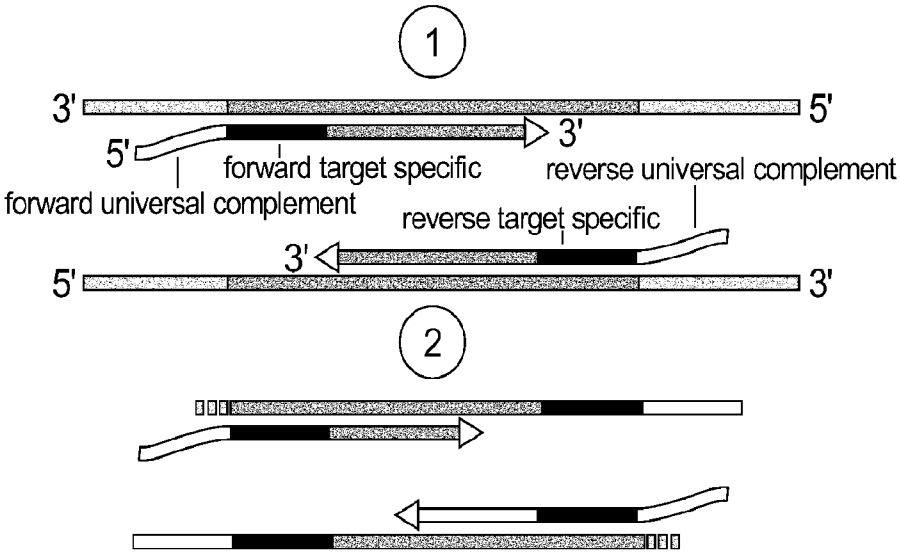


Distinct viral types are differentiated by amplicon size. Specificity is conferred by priming sites at evolutionarily divergent sequence regions.

Multiplexed chimeric primers
3' (black) specific to one viral type
5' (blue) universal priming sequences

Above, 3 of 6 possible viral types are in one cell population.

Cycles 1 and 2 incorporate universal priming sequences via target-specific 3' ends of chimeric primers.



Cycle 3 begins incorporation of universal primers with WeiiRED 04 fluorescent tag.

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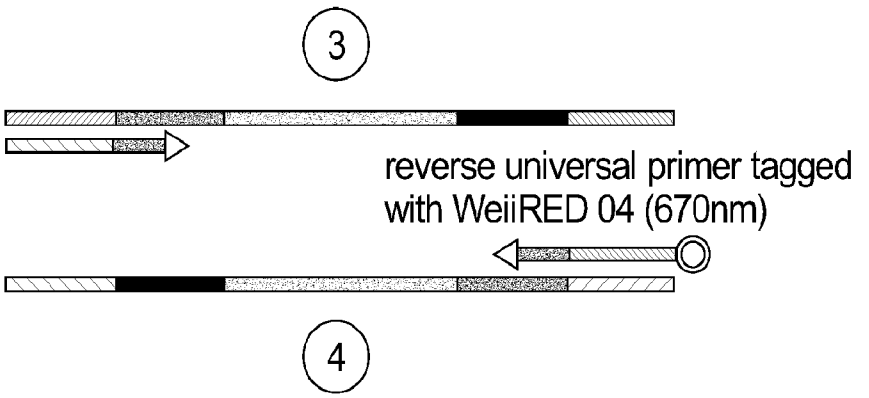
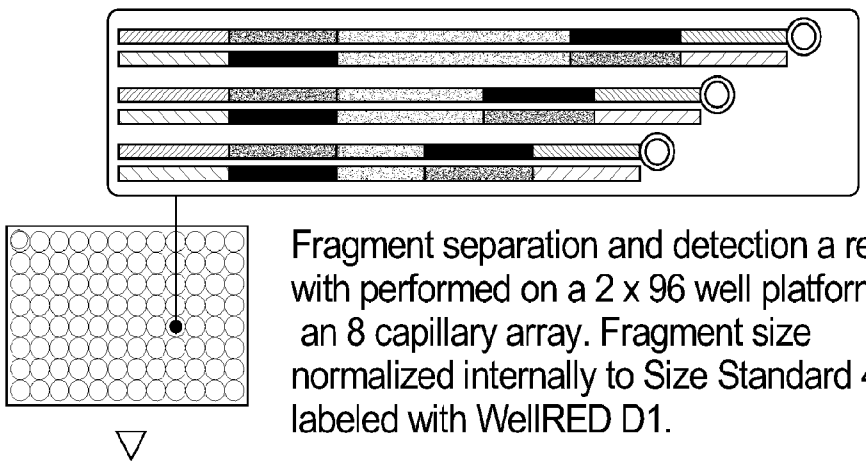
FIG. 2 cont.



Subsequent cycles produce amplicons of three sizes representing the viral types present in the cell population above.



Fragment separation and detection a re with performed on a 2 x 96 well platform with an 8 capillary array. Fragment size normalized internally to Size Standard 400 labeled with WellRED D1.

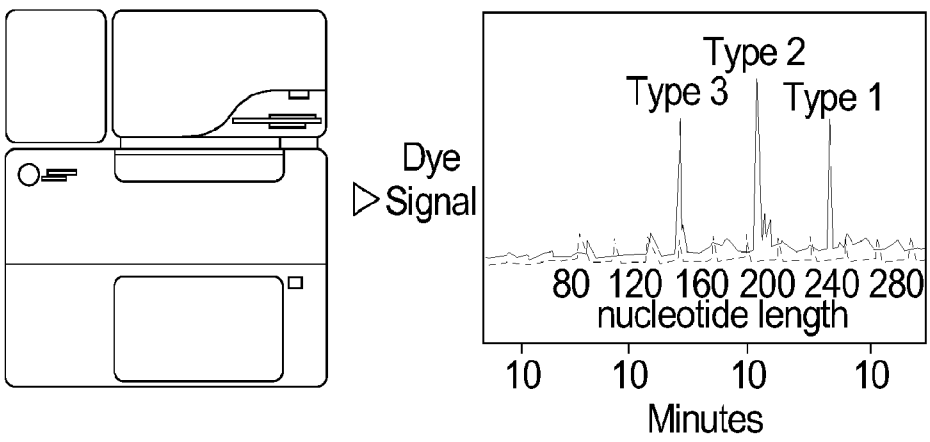


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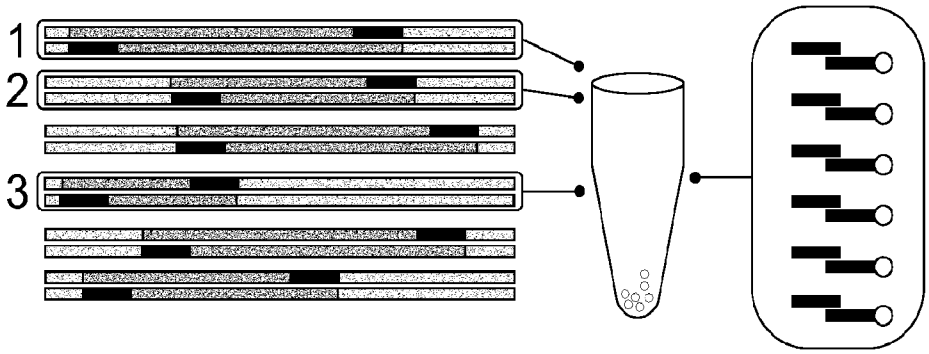
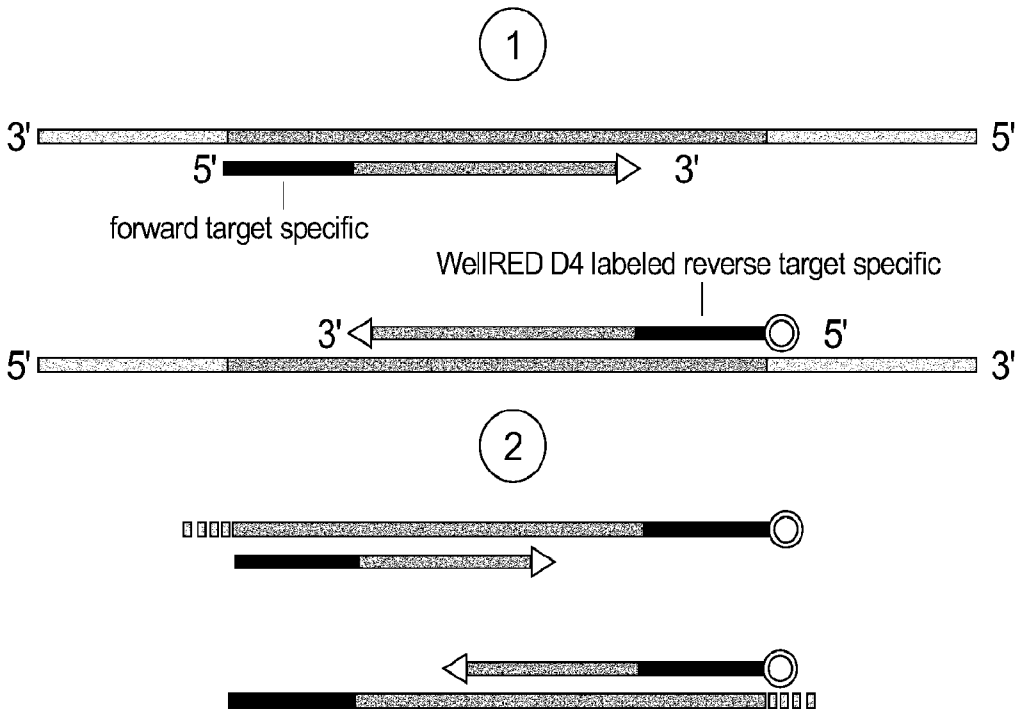
FIG. 2 cont.

Labeled HPV primer strategy



An alternate strategy produces fluorescence tagged amplicons by having one primer of each pair 5' end-labeled with WeiiRED D4.

Again, an example is shown in which 3 of 6 viral types are present in the sample of sorted cells.



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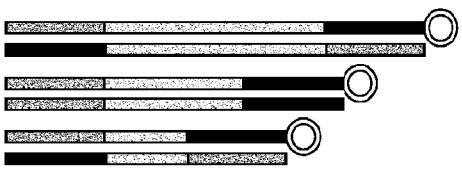
from sheet 4/13

FIG. 2 cont.

3

Subsequent cycles produce amplicons representing the viral types present in the cell population above.



Fragment separation and detection are performed using the GeXP platform as shown at left.

Figure 2. PCR primer labeling and amlification strategies. At left, amplicons have fluorescent tags as a result of labeled universal primers. At right, each pair of HPV typespecific primers has one member of the pair labeled. In both cases, fragment separation and detection are performed using automated capillary electrophoresis.

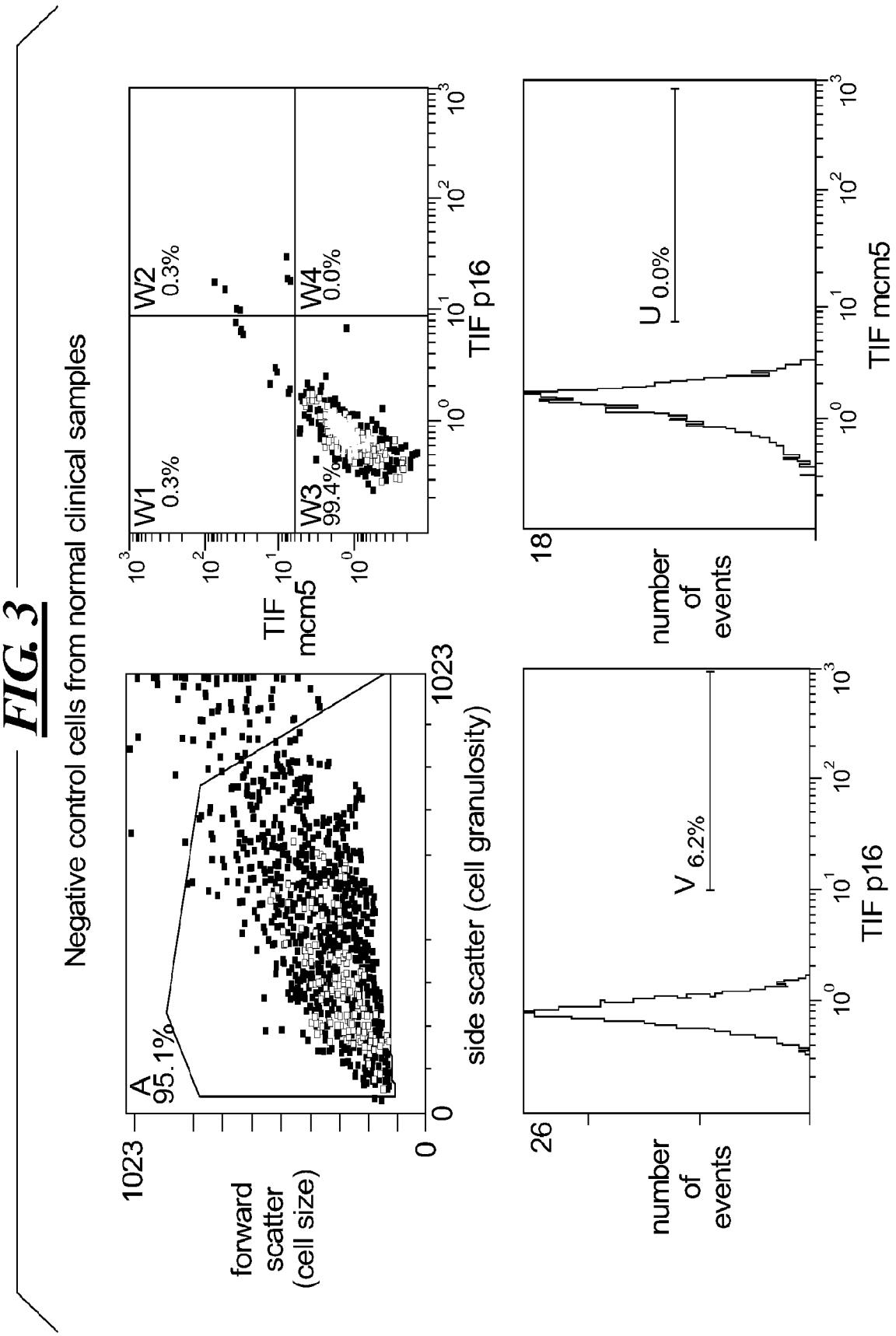

Negative control cells from normal clinical samples
A
95.1%
forward scatter (cell size)
side scatter (cell granulosity)
1023
0
W1
0.3%
W2
0.3%
W3
99.4%
W4
0.0%
TIF mcm5
TIF p16
26
number of events
V 6.2%
TIF p16
18
U 0.0%
TIF mcm5

FIG. 3

FIG. 4

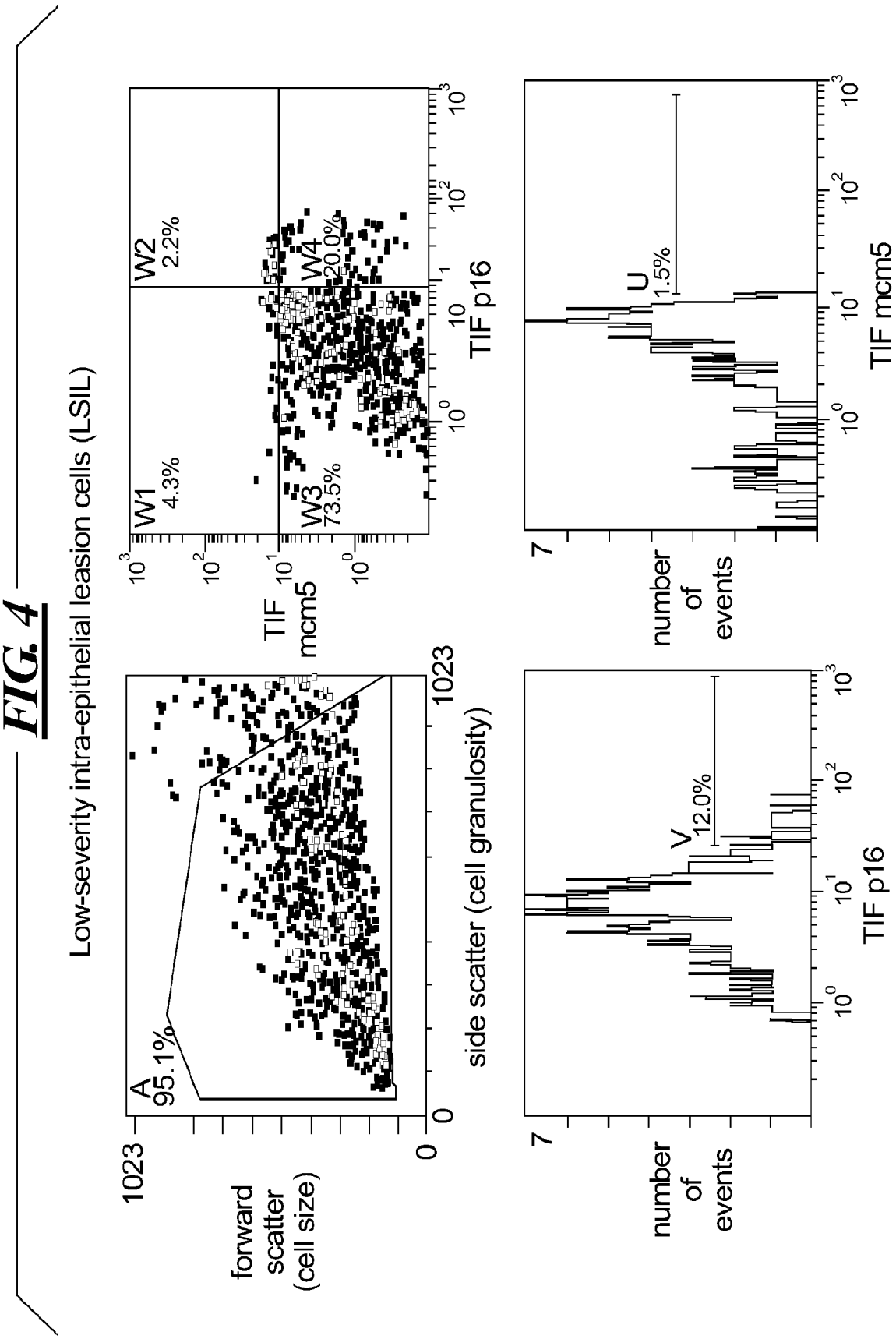
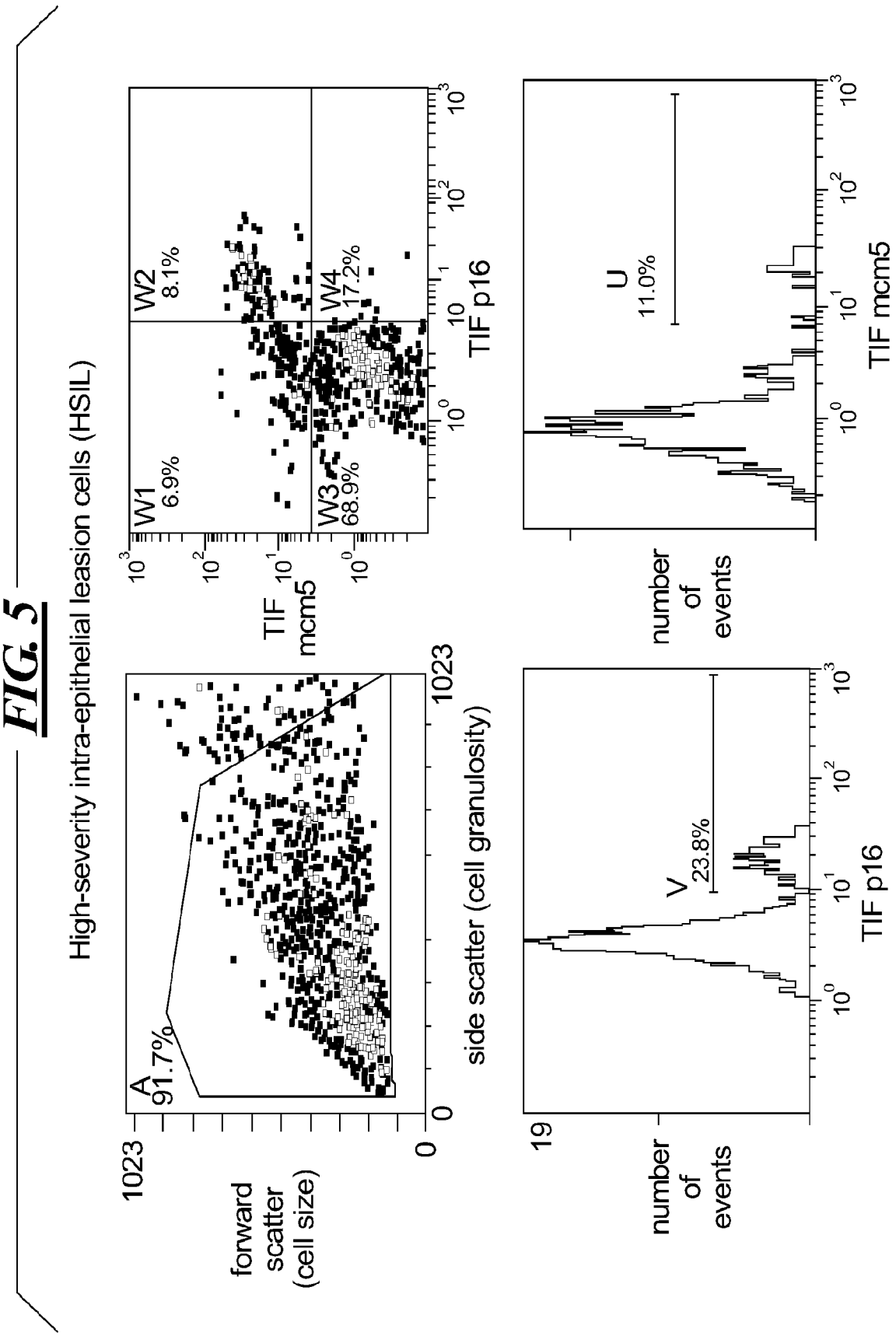

Low-severity intra-epithelial leasion cells (LSIL)
forward scatter (cell size)
side scatter (cell granulosity)
A
95.1%
1023
0
0
1023
TIF mcm5
TIF p16
W1
4.3%
W2
2.2%
W3
73.5%
W4
20.0%
number of events
TIF p16
V
12.0%
number of events
TIF mcm5
U
1.5%

FIG. 5


High-severity intra-epithelial leasion cells (HSIL)
forward scatter (cell size)
side scatter (cell granulosity)
A
91.7%
1023
0
0
1023
TIF mcm5
TIF p16
W1
6.9%
W2
8.1%
W3
68.9%
W4
17.2%
number of events
TIF mcm5
U
11.0%
number of events
TIF p16
V
23.8%
19

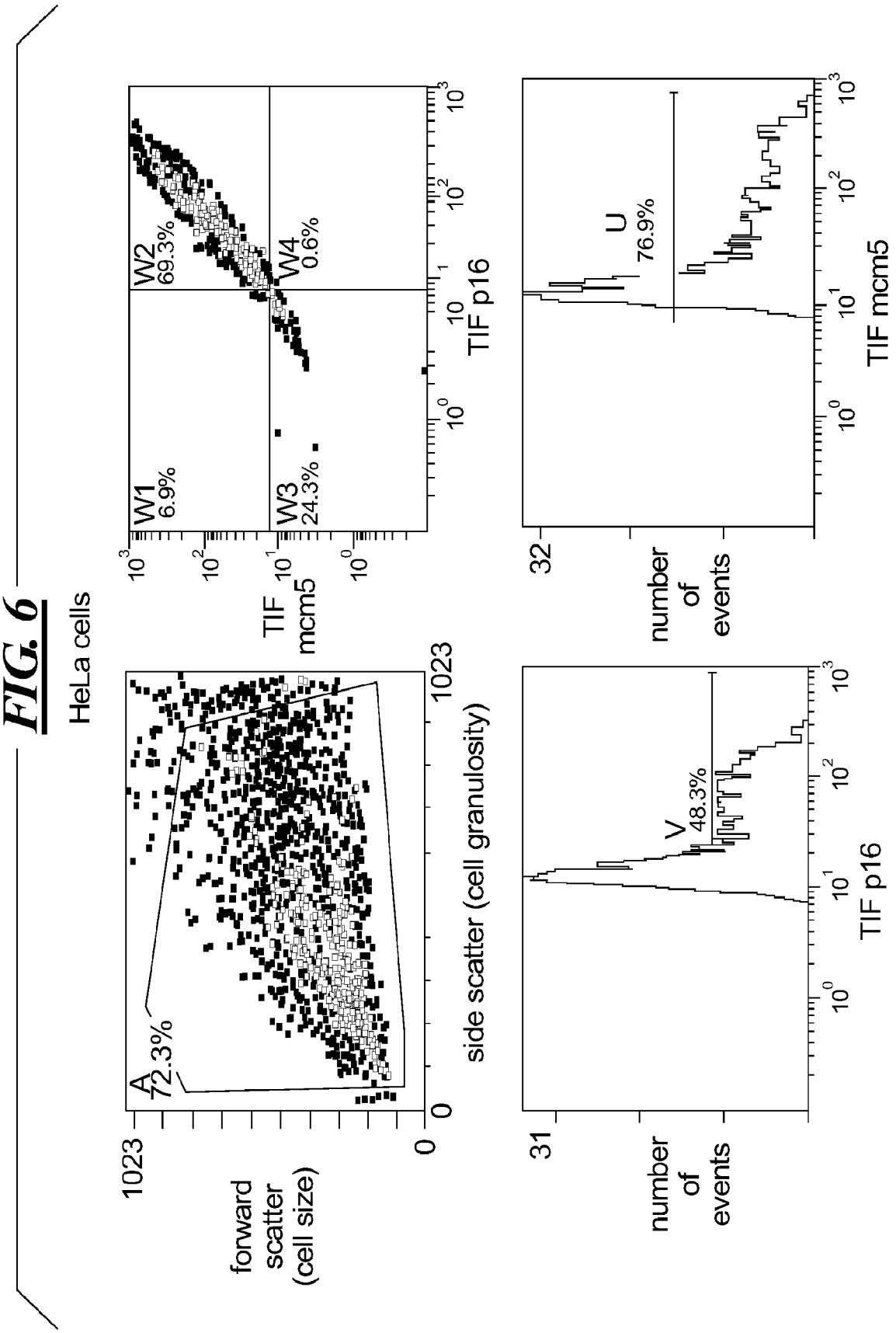

HeLa cells
forward scatter (cell size)
side scatter (cell granulosity)
A
72.3%
1023
0
TIF mcm5
TIF p16
W1
6.9%
W2
69.3%
W3
24.3%
W4
0.6%
number of events
U
76.9%
V
48.3%
31
32

FIG. 6

FIG. 7

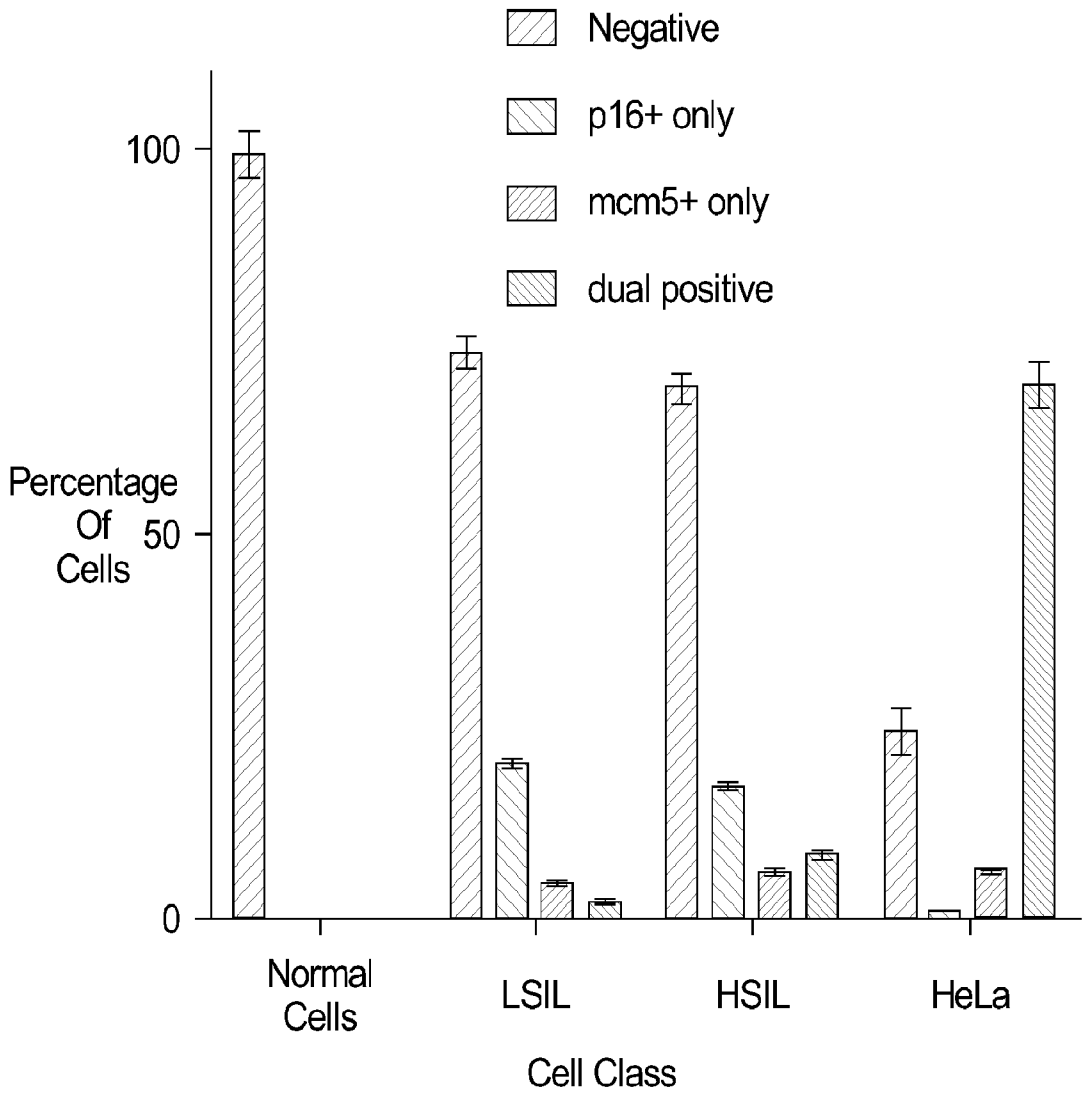
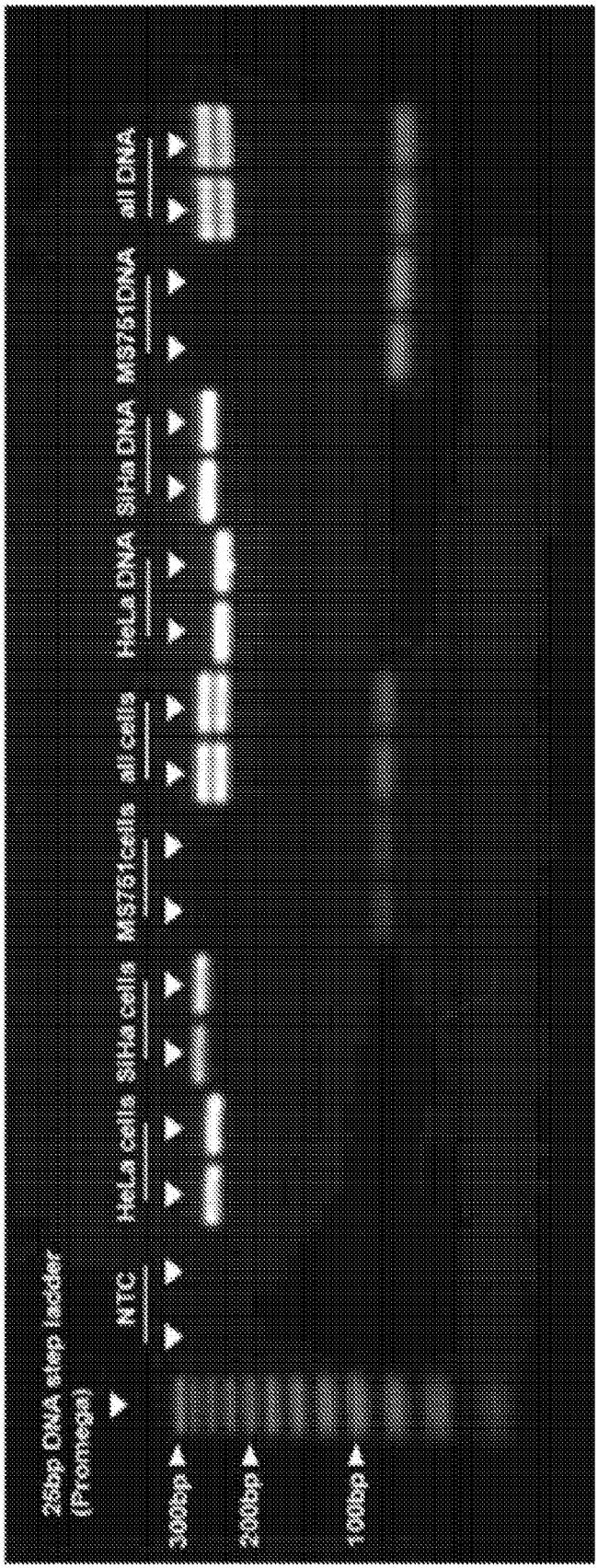

Percent signal type by sample class.
Negative
p16+ only
mcm5+ only
dual positive
100
50
0
Percentage Of Cells
Normal Cells
LSIL
HSIL
HeLa
Cell Class

FIG. 8



Gel electrophoresis of HPV 16, 18 and 45 amplicons as proof of concept for genotype specificity and sensitivity from sorted cells.

FIG. 9

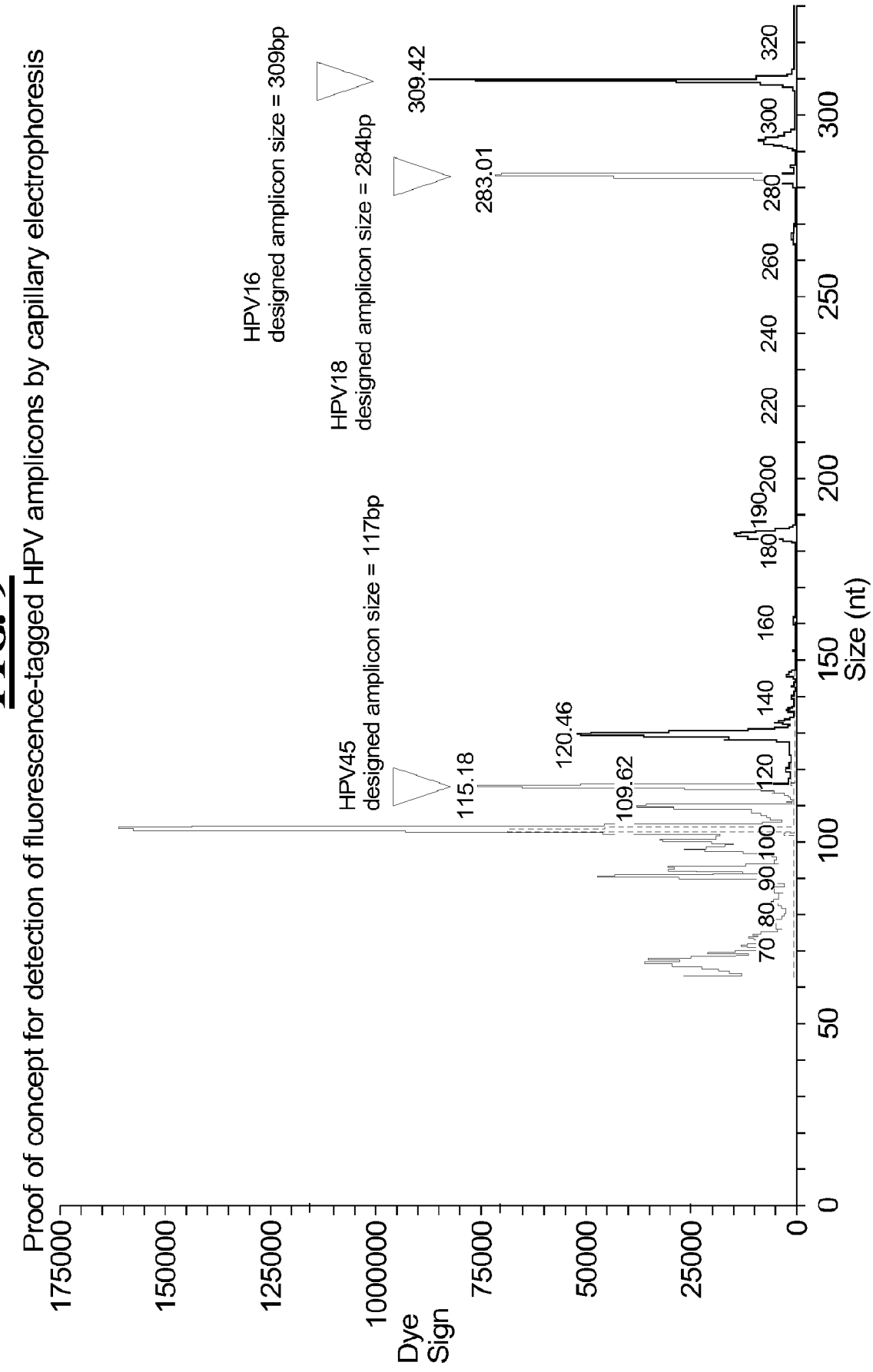
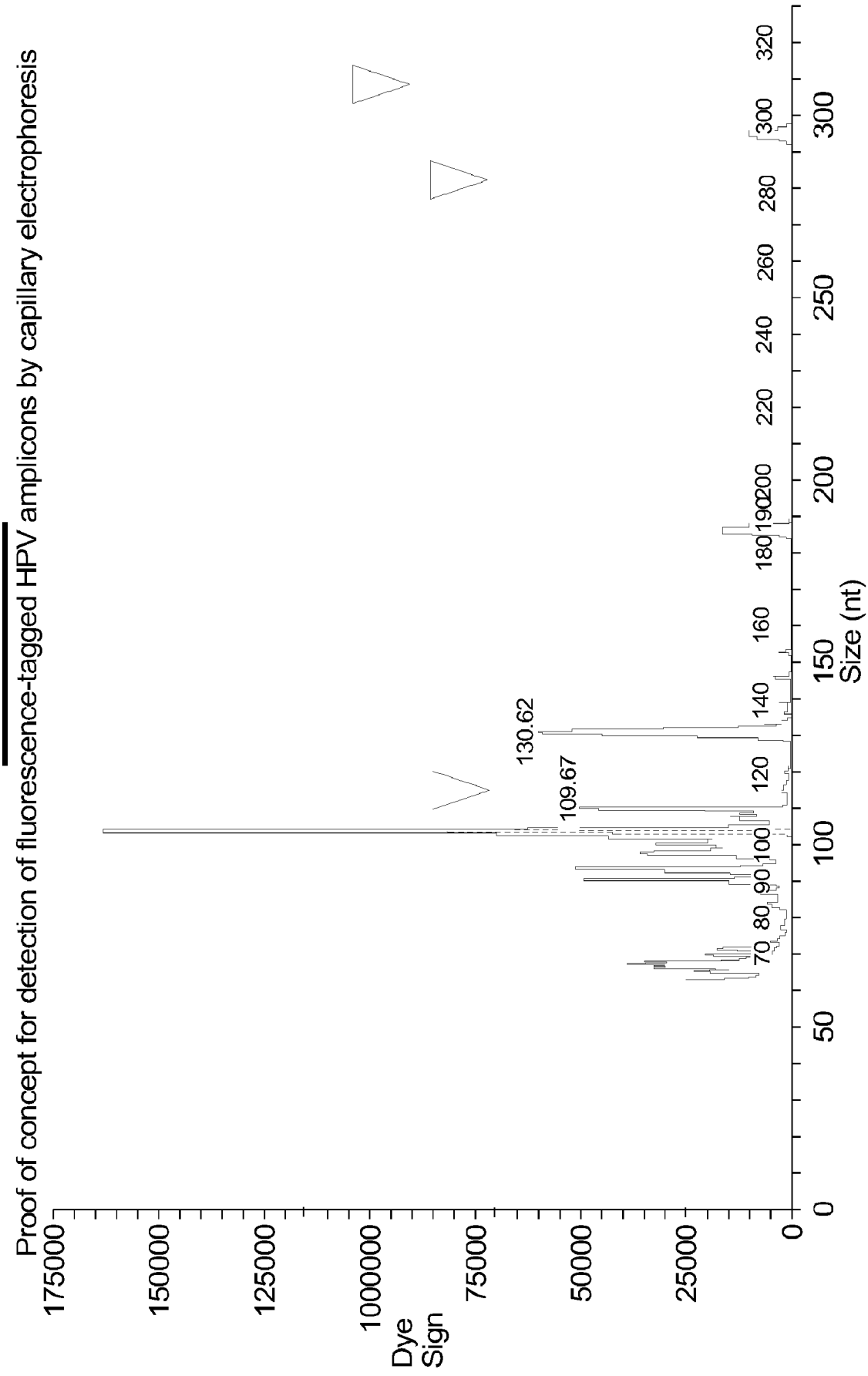

Proof of concept for detection of fluorescence-tagged HPV amplicons by capillary electrophoresis
HPV45
designed amplicon size = 117bp
HPV18
designed amplicon size = 284bp
HPV16
designed amplicon size = 309bp
115.18
109.62
120.46
283.01
309.42
Dye
Sign
Size (nt)
175000
150000
125000
1000000
75000
50000
25000
0
0
50
100
150
200
250
300
70
80
90
100
120
140
160
180
190
200
220
240
260
280
300
320

FIG. 9 cont.


Proof of concept for detection of fluorescence-tagged HPV amplicons by capillary electrophoresis
Dye Sign
Size (nt)
130.62
109.67
175000
150000
125000
1000000
75000
50000
25000
0
0
50
100
150
200
250
300
70
80
90
100
120
140
160
180
190
200
220
240
260
280
300
320