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(54) **Title**: NOVEL MONOCLONAL ANTIBODIES AGAINST HPV PROTEINS

(57) **Abstract**: Embodiments of the invention provide methods, monoclonal antibodies, polyclonal antibodies, assays, and kits for detecting HPV infection and HPV related cancer diagnosis, including infection by various HPV genotypes, early and/or late stage HPV-associated or HPV-specific cancers. Various monoclonal antibodies recognizing specific epitope for specific HPV protein or HPV type, common epitope for various HPV proteins or HPV types are obtained. The obtained monoclonal antibodies are useful tools in early clinical detection of HPV infection and general detection of HPV related diseases, specific detection of invasive cervical cancer, detection of other HPV related cancers, early stage precancerous lesions as well as late stage cancer progression.

Attorney Docket No.: NEOD/0004 PCT

NOVEL MONOCLONAL ANTIBODIES AGAINST HPV PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of United States provisional patent application serial number 61/131,991, filed June 13, 2008, and Uinted States provisional patent application Serial No. 61/192,912, filed September 22, 2008. Each of the aforementioned related patent applications is herein incorporated by reference.

BACKGROUND OF THE INVENTION.

[0002] Infection by human papillomaviruses (HPV) at specific epithelium cells to induce epithelial proliferations plays an important role for cervical carcinogenesis. About 99 percent of confirmed cervical cancer cases are found to be associated with HPV infection with biopsy-confirmed squamous intraepithelial lesions (SIL) or cervical intraepithelial neoplasia (CIN). Approximately 1% of the population has genital warts and 4% of women have cervical precancerous lesions, such as low grade of squamous intraepithelial lesion (LSIL) or high grade of squamous intraepithelial lesion (HSIL). or atypical squamous cells of undetermined significance (ASCUS). It is general thought that persistent infection of human papillomavirus (HPV) is essential for developing precancerous epitheliual lesions. Infection of high-risk types HPV for women with LSIL may or may not progress to HSIL. In fact, remission occurs in majority of LSIL human subjects while some progress to HSIL. Although 99.7% of cervical cancers are HPV positive, integration of viral genome into the host genome is required to facilitate the necessary genes to express for developing into HSIL or cancer. Only one in every 10 women with persistent HPV infection may develop into higher grades of CIN lesions, such as cervical intraepithelial neoplasia (CIN) grade 2 and grade 3 (CIN2, and CIN3, respectively), and a portion of these epitheliual lesion cases may ultimately progress into cervical cancer.

[0003] In the past, screening for cervical cancer is based on conventional cytology screening tests, e.g., obtaining papanicolaou (Pap) smears for cytological staining tests, and suspicious smears are followed up with colposcopy, and/or histological biopsy. However, due to subjective test criteria, there are various drawbacks for pap smear tests: difficulty in obtaining samples, poor inter- and intra-observer agreement, high rates of false negatives nd false positives, requiring specialized labs staffed with highly trained personnel, and inability to identify the majority of HPV-infected human subjects. More reproducible assays are needed to improve the current screening tests to avoid unnecessary medical intervention and psychological distress for the affected women.

[0004] Detecting HPV infection by nucleic acid tests, such as "DNA Hybrid Capture", has been developed with high assay sensitivity, but are still not ideal, due to not only its high cost, assay operation procedures, the requirements for facility, equipment, and highly trained personnel, but also its very low positive predictive value (PPV) in cervical intraepithelial neoplasia (CIN) testing samples. Assay like PreTect HPV-Proofer® provides the detection of E6/E7 mRNA with sensitivity equivalent to HPV Hybrid Capture tests with higher positive predictive value; but cannot directly detect E6/E7 oncoprtoeins *in situ*. In addition, DNA testing could not differentiate disease stages after HPV infection nor the diagnosis of different cell lesions (e.g., cannot diffrentiate LSIL from HSIL, nor CIN lesions from non-transforming latent or remissive viral infection). What is needed is a low cost, simple, sensitive and specific assay that can be performed on routine practice of a clinical lab or doctor office and capable of detecting early stages of epithelial lesions, distinguish LSIL from HSIL, or predicting the risk of progression into cervical cancer.

[0005] Known protocols for producing monoclonal antibodies are generally unsuitable for the production of anti-HPV monoclonal antibodies and cannot be used in immunocytochemical diagnostic tests performed on human subjects of general population. This is because antibodies produced by these protocols will not necessarily react with the naturally occurring HPV viral proteins in infected human

cells. In addition, three problems exist in clinical HPV detection. One is that HPV proteins in clinical samples are present in very small quantities. Secondly, there are too many HPV types and most HPV types present in clinical samples are not known or systemically identified due to the lack of avaialbe antibodies. Third, HPV virus can not be cultured in labs by standard tissue culture techniques. Thus, there is no avaialbe HPV proteins purified to large quantities as immunogens for generating anti-HPV antibodies, and there is no avaialbe HPV proteins or purified anti-HPV antibodies to recognize anti-viral antibodies or viral proteins present in clinical samples for clinical HPV detection.

[0006] Infections by only about 15 HPV types (out of more than 100 available HPV types) are at high risk of developing into cervical intraepithelial neoplasia (CIN) or cervical cancer. Among them, around 70% of reported cervical cancer cases and 50% of reported CIN 2 and CIN 3 cases are caused by two high risk HPV types, i.e., HPV type-16 and HPV type-18. However, some progressive cervical cancer cases are reported to be infected by low risk HPV types, while infection of some high risk HPV types will never progress into cervical cancer. Infections by these two prevailing high risk HPV types do not correlate with tumor development or cancer progression. It seems important to identify those HPV-infected human subjects that express particular oncogenic proteins rather than just identify HPV infection by high risk types.

[0007] Thus, there is a need for detect the expression of HPV-related oncoproteins in clinical samples as these oncoprotiens may be better serve as cervical cancer biomarkers to better predict the risk in developing into high grade of cell lesions or cervical cancer-related diseases. There is also a need to develop anti-HPV antibodies and appropriate HPV immunoassays to detect the presence of invasive cervical cancer and/or HPV-related oncoproteins as cervical cancer biomarkers and predict the risk for malignant transformation of epithelial lesions into cervical cancer.

SUMMARY OF THE INVENTION

[0008] Embodiments of the invention generally relate to various methods, detection assays, kits, polyclonal and monoclonal anti-HPV antibodies, polypeptides, recombinant HPV proteins, and nucleic acids useful for detecting HPV infection, including general HPV infection as well as infection by various HPV genotypes, high risk HPVs and low risk HPVs. Various novel monoclonal antibodies against HPV proteins, useful as biomarkers and useful tools for detecting HPV viral proteins, HPV oncoproteins, early screening of cervical cancer, and diagnosing CIN, dysplasia stages, and/or invasive cervical and other cancers, are provided. The tools of the inventions can also be used in early clinical detection for HPV infection and general diagnosis for cervical cancer and other cancers, specific detection of invasive cervical cancer, detection of other HPV related cancers, early stage precancerous lesions as well as late stage cancer progression.

[0009] Methods of producing the monoclonal antibody are provided herein to obtain monoclonal antibodies recognizing one or more common epitopes of HPV proteins among various HPV proteins or HPV types, e.g., pan anti-HPV antibody. In addition, some of the monoclonal antibodies obtained herein are HPV type-specific, while some of the monoclonal antibodies obtained herein are non-HPV type-specific. The non-HPV-type-specific pan antibodies recognize and bind to HPV proteins from most prevalent HPV types present in clinical samples. In one aspect of the invention, the binding capabilities of these pan monoclonal antibodies are suitable to be used in a single convenient assay to detect HPV infection in one or more clinical samples. In anther aspect, two or more HPV immunological assays can also be used. In another aspect, anti-HPV antibodies with specificity to a single HPV protein (only a single HPV protein from a single HPV type, but not other HPV types, not other HV proteins) are also obtained.

[0010] One embodiment of the invention provides a monoclonal antibody obtained by screening antibody-producing hybridoma cells with two or more

purified recombinant human papillomavirus viral proteins. The screening step includes selecting the antibody-producing hybridoma cells with positive reactivity to the two or more purified recombinant papillommavirus proteins and with negative reactivity to non-HPV proteins, such that the antibody-producing hybridoma cells generate the monoclonal antibody with binding specificity to two or more human papillomavirus viral proteins. The various HPV associated antibodies may include polyclonal and monoclonal antibodies which show specificity for one or more HPV proteins encoded by early genes and/or late genes from one HPV genotype or two or more genotypes. The antibodies described herein can be used to perform immunoassays for different human subjects and compare with positive and negative controls.

[0011] In one embodiment, a monoclonal antibody is obtained by screening antibody-producing hybridoma cells with a first purified recombinant human papillomavirus protein from a first HPV type and a second purified recombinant human papillomavirus protein from a second HPV type such that the monoclonal antibody is capable of recognizing a common epitope on human papillomavirus viral proteins from the same or different HPV types. In another embodiment, a monoclonal antibody, obtained by screening antibody-producing hybridoma cells with a first purified recombinant human papillomavirus protein from a first HPV type and a second purified recombinant human papillomavirus protein from a second HPV type such that the monoclonal antibody is capable of recognizing a specific epitope on only one of the first and the second purified recombinant human papillomavirus proteins and not the other purified recombinant human papillomavirus protein, is provided.

[0012] In another embodiment, a monoclonal antibody capable of binding to two or more HPV viral proteins from the same HPV type is provided and obtained by screening antibody-producing hybridoma cells with two or more purified recombinant papillomavirus proteins. The two or more purified recombinant papillomavirus proteins can be from the same HPV type and/or from different HPV types, such that the antibody-producing hybridoma cells generate the monoclonal

antibody with binding specificity to two or more human papillomavirus viral proteins from the same types. The two or more purified recombinant papillomavirus proteins can be two or more purified recombinant papillomavirus early proteins such that the monoclonal antibody is capable of binding to two or more HPV early proteins corresponding to the two or more purified recombinant papillomavirus early proteins. The two or more purified recombinant papillomavirus proteins comprises a purified recombinant papillomavirus early protein and a purified recombinant papillomavirus late protein such that the monoclonal antibody is capable of binding to an early viral protein and a late viral proteins corresponding to the purified recombinant papillomavirus early protein and the purified recombinant papillomavirus late protein.

[0013] In another embodiment, a monoclonal antibody capable of binding to two or more HPV viral proteins from different HPV types is provided. Such a monoclonal antibody is obtained by screening antibody-producing hybridoma cells with two or more purified recombinant papillomavirus proteins, wherein the screening step includes selecting the antibody-producing hybridoma cells with positive reactivity to the two or more purified recombinant papillommavirus proteins from different HPV types and with negative reactivity to non-HPV proteins, such that the antibody-producing hybridoma cells generate the monoclonal antibody with binding specificity to the two or more HPV viral proteins.

[0014] In still another embodiment, a monoclonal antibody capable of binding to an early HPV viral protein and a late HPV viral protein from different HPV types is provided. In yet another embodiment, a monoclonal antibody capable of binding to only a first HPV viral protein, but not a second HPV viral protein different from the first HPV viral protein, and obtained by screening antibody-producing hybridoma cells with positive reactivity to a first purified recombinant papillomavirus protein from a first HPV type and with negative reactivity to a second purified recombinant papillomavirus protein from a second HPV type, wherein the first and second viral proteins correspond to the first and the second purified recombinant papillomavirus proteins of the first and second HPV types.

Attorney Docket No.: NEOD/0004 PCT

SUMMARY OF DRAWING:

[0015] Figure 1A shows the specificity of a monoclonal antibody capable of reacting with both HPV16 E6 and HPV16 E7 recombinant proteins (different HPV proterins from the same HPV type) and recognizing a common epitope on the different HPV16 E6 and HPV16 E7 proteins from the same HPV 16 type as assayed on EIA (enzyme immuno assays) according to one embodiment of the invention.

[0016] Figure 1B shows the specificity of another monoclonal antibody capable of reacting with both HPV16 E6 and HPV16 E7 recombinant proteins and recognizing a common epitope on the HPV 16 E6 and HPV16 E7 proteins as assayed on EIA according to one embodiment of the invention.

[0017] Figure 2A shows the specificity of a monoclonal antibody capable of reacting with HPV16 E6, E7, L1 & L1 N-terminal recombinant proteins (different HPV proterins from the same HPV type) and recognizing a common epitope on the different E6, E7, L1, and L1 N-terminal proteins from the same HPV 16 type as assayed on EIA according to another embodiment of the invention.

[0018] Figure 2B shows a western blot of the monoclonal antibody as shown in Figure 2B, confirming its binding to all of the HPV16 E6, E7 and L1 recombinant proteins.

[0019] Figure 2C shows the results of a western blot of cell lysate from cervical cancer cell lines using the monoclonal antibody as shown in Figure 2A, confirming its binding to all of the HPV16 E6, E7 and L1 viral proteins present in these cervical cancer cell lines.

[0020] Figure 3A shows the specificity of a monoclonal antibody capable of binding to all of the recombinant HPV16 E6, E7, L1 N-terminal proteins as well as HPV18 E6 and E7 proteins (HPV proterins from different HPV types) and

recognizing a common epitope on the E6, E7, L1 N-terminal proteins from HPV16 and HPV18 as assayed on EIA according to another embodiment of the invention.

[0021] Figure 3B shows the results of a western blot of the monoclonal antibody as shown in Figure 3A, confirming its binding to the different recombinant proteins and recognizing a common epitope on the different E6, E7 and L1-N terminal proteins from the two different HPV types HPV16 and HPV18.

[0022] Figure 3C shows a western blot cell lysate from cervical cancer cell lines using the monoclonal antibody as shown in Figure 3A, confirming its binding to the HPV16 E6, E7 and L1 proteins as well as HPV18 E6, E7 and L1-N terminal viral proteins present in these cervical cancer cell lines.

[0023] Figure 4A shows the specificity of a monoclonal antibody capable of binding to two E6 recombinant proteins (HPV16 E6 and HPV18 E6, E6 proterins from different HPV types) and recognizing a common epitope on the two E6 proteins from different HPV types as assayed on EIA according to another embodiment of the invention.

[0024] Figure 4B shows the results of a western blot analyzing the cell lysate from cervical cancer cell lines using the monoclonal antibody as shown in Figure 4A, confirming its binding to HPV16 E6 as well as HPV18 E6 viral proteins present in these cervical cancer cell lines.

[0025] Figure 5 shows the specificity of a monoclonal antibody capable of reacting with two recombinant HPV16 E7 and HPV18 E7 proteins (E7 proterins from different HPV types) and recognizing a common epitope on the two E7 proteins from different HPV types as assayed on EIA.

[0026] Figure 6 shows the specificity of a monoclonal antibody capable of reacting with only HPV16 E6 recombinant protein but not with any other HPV recombinant proteins on EIA according to one embodiment of the invention.

[0027] Figure 7A shows the specificity of a monoclonal antibody capable of reacting specifically with only HPV18 E6 recombinant protein, but not with any other HPV16 or HPV18 recombinant proteins as assayed on EIA according to another embodiment of the invention.

[0028] Figure 7B shows the results of a western blot analyzing the cell lysate from different cervical cancer cell lines using the monoclonal antibody as shown in Figure 7A, confirming its binding to the-HPV18 E6 viral protein but not HPV E7 viral protein that are present in Hela cancer cell line.

[0029] Figure 8 shows the specificity of a monoclonal antibody capable of reacting specifically with a HPV16 E7 recombinant protein, but not with any other HPV recombinant proteins as assayed on EIA according to another embodiment of the invention.

[0030] Figure 9 shows the specificity of a monoclonal antibody capable of reacting specifically with a recombinant HPV18 E7 recombinant protein, but not with any other HPV recombinant proteins as assayed on EIA according to another embodiment of the invention.

[0031] Figure 10A shows the specificity of a monoclonal antibody capable of reacting specifically with a HPV16 L1 N-terminal recombinant protein, but not with any other HPV recombinant proteins as assayed on EIA according to another embodiment of the invention.

[0032] Figure 10B shows the specificity of another monoclonal antibody capable of reacting specifically with a HPV16 L1 N-terminal recombinant protein, but not with any other HPV recombinant proteins as assayed on EIA.

[0033] Figure 11 shows the specificity of a monoclonal antibody capable of reacting specifically with only the HPV16 L1 & L1 N-termianl recombinant proteins, but not with any other HPV recombinant proteins as assayed on EIA according to another embodiment of the invention.

[0034] Figure 12A shows the representative staining image of the dysplasia cells of CIN2 tissues using an anti-E6 monolonal antibody in an immunohistocytostaining (IHC) assay according to one embodiment of the invention.

[0035] Figure 12B shows the representative staining image of the adjacent normal epithelium from the dysplasia tissue of the CIN2 sample of Figure 12A according to another embodiment of the invention.

[0036] Figure 12C shows the representative staining image of the dysplasia epithelium of a CIN3 sample stained by the same anti-E6 monolonal antibody as used in Figure 12A in an IHC assay, demonstrating specific IHC staining in the nuclear and cytoplasm of dysplasia cells by the anti-E6 monoclonal antibody according to another embodiment of the invention.

[0037] Figure 12D shows the representative staining image of the dysplasia epithelium of another CIN3 sample stained by the same anti-E6 monolonal antibody as used in Figure 12A in an IHC assay.

[0038] Figure 13A shows the representative staining image of a squamocarcinoma (SCC) tissue from tissue microarray using an anti-E7 monolonal antibody in an immunohistocytostaining (IHC) assay according to another embodiment of the invention.

[0039] Figure 13B shows the representative staining image of the normal epithelium (about 15 mm away from the tumor tissue) adjacent the SCC tissue of Figure 13A.

[0040] Figure 13C shows the representative staining image of another SCC tisse sample stained by the same anti-E7 monolonal antibody as used in Figure 13A in an IHC assay, demonstrating specific IHC staining in the tumor cells by the anti-E7 monoclonal antibody.

[0041] Figure 13D shows the magnified representative image of of the tumor cells from Figure 13C to view the staining of the cytoplasm of the tumor cells.

[0042] Figure 14A shows the representative staining image of cervical cells from a CIN2 cervical scrape sample and stained by a mouse monoclonal anti-HPV E7 antibody in an immunocytochemistry (ICC) assay according to one embodiment of the invention.

[0043] Figure 14B shows the representative staining image of cervical cells from a CIN3 cervical scrape sample and stained by a mouse monoclonal anti-E6 antibody in an ICC assay according to another embodiment of the invention.

[0044] Figure 14C shows the representative image of cervical cells from an adenocarcinoma (ADC) cervical scrape sample and stained by the same anti-E6 antibody shown in Figure 14B in an ICC assay.

DETAILED DESCRIPTION

[0045] Embodiments of the invention provide various monoclonal antibodies against HPV proteins such that infection by high risk and low risk HPV types can be detected by a single monoclonal antibody. The invention also provides HPV type specific monoclonal antibodies for detecting only the high risk HPV types. In addition, monoclonal antibodies highly specific for a single HPV protein are also provided.

[0046] One aspect of the invention provides a method of producing monoclonal antibodies. The method includes obtaining various purified recombinant papillomavirus proteins and screening antibody-producing hybridoma cells with two or more purified recombinant papillomavirus proteins to obtain a monoclonal antibody capable of recognizing a common epitope on the two or more purified recombinant human papillomavirus proteins and binding to the two or more purified recombinant papillomavirus proteins and corresponding papillomavirus viral proteins in biological and clinical samples.

[0047] In addition, the monoclonal antibody with binding specificity to two or more human papillomavirus viral proteins is produced by using a method which includes positive selection of the antibody-producing hybridoma cells with two or more purified recombinant papillommavirus proteins and negative selection of the antibody-producing hybridoma cells with non-HPV proteins. For example, the method may include screening antibody-producing hybridoma cells with two or more purified recombinant papillomavirus proteins by selecting the antibody-producing hybridoma cells with positive reactivity to the two or more purified recombinant papillommavirus proteins and with negative reactivity to non-HPV proteins, such that the antibody-producing hybridoma cells generate the monoclonal antibody with binding specificity to the two or more human papillomavirus viral proteins. The two or more purified recombinant papillomavirus proteins may include, for example, HPV 16 E6 protein, HPV 16 E7 protein, HPV 16 L1 protein, HPV 18 E6 protein, HPV18 E7 protein, HPV 18 L1 protein, and combinations thereof.

[0048] Another method of the invention includes screening antibody-producing hybridoma cells with a first purified recombinant human papillomavirus protein from a first HPV type and a second purified recombinant human papillomavirus protein from a second HPV type to obtain a monoclonal antibody capable of recognizing a common epitope on human papillomavirus proteins from two or more different HPV types. Still, another method of the invention provides screening antibody-producing hybridoma cells with a first purified recombinant human papillomavirus protein from a first HPV type and a second purified recombinant human papillomavirus protein from a second HPV type to obtain a monoclonal antibody capable of recognizing a specific epitope on only one of the first and the second purified recombinant human papillomavirus proteins and not the other purified recombinant human papillomavirus proteins.

[0049] For example, a monoclonal antibody capable of binding to two or more HPV viral proteins from the same HPV type is produced by the method of the invention by screening antibody-producing hybridoma cells with two or more

purified recombinant papillomavirus proteins from the same HPV type and/or from different HPV types. In one example, the two or more purified recombinant papillomavirus viral proteins are two or more purified recombinant papillomavirus early proteins such that a monoclonal antibody is produced and is capable of binding to two or more HPV early proteins corresponding to the two or more purified recombinant papillomavirus early proteins. In another example, the two or more purified recombinant papillomavirus proteins may include a purified recombinant papillomavirus early protein and a purified recombinant papillomavirus late protein such that another monoclonal antibody is produced and is capable of binding to an early viral protein and a late viral proteins corresponding to the purified recombinant papillomavirus early protein and the purified recombinant papillomavirus late protein.

[0050] Exemplary monoclonal antibodies obtained include a type of monoclonal antibody capable of binding to both HPV16 E6 and HPV16 E7 viral proteins; another type of monoclonal antibody capable of binding to all HPV16 E6, HPV16 E7, and HPV16 L1 viral proteins; and anther type of monoclonal antibody capable of binding to both HPV18 E6 and HPV18 E7 viral proteins. Accordingly, the monoclonal antibody produced using methods of the invention is capable of binding to the two or more HPV viral proteins from the same HPV type selected from the group consisting of high risk HPV types, low risk HPV types, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68, HPV-6, HPV-11, HPV-42, HPV-43, HPV-44, HPV-53, HPV-54, HPV-55, and HPV-56, and combinations thereof.

[0051] These monoclonal antibodies can be used for one or more immunological assays to detect HPV infection and HPV-related cervical cancer and other diseases. The suitable immunological assay may include ELISA (enzyme linked immunoabsorbant assays), antigen assays for papillomavirus proteins, antibody assays for antibodies against papillomavirus proteins, assays for papillomavirus immunocomplexes, protein chip assays, radioimmunoprecipitation assays, rapid membrane immunochromatographic assays, rapid stick

immunochromatographic assays, immunohistochemistry for tissues and/or cervical cells, and immunocytochemistry assays followed by flow cytometry.

[0052] Another aspect of the invention provides a method and a monoclonal antibody capable of binding to two or more HPV viral proteins from different HPV types. The monoclonal antibody is obtained by screening antibody-producing hybridoma cells with two or more purified recombinant papillomavirus proteins by selecting the antibody-producing hybridoma cells with positive reactivity to the two or more purified recombinant papillommavirus proteins from different HPV types and with negative reactivity to non-HPV proteins, such that the antibody-producing hybridoma cells generate the monoclonal antibody with binding specificity to the two or more HPV viral proteins. Exemplary monoclonal antibodies include a type of monoclonal antibody capable of binding to HPV16 E7 and HPV18 E7 proteins; another type of monoclonal antibody capable of binding to HPV16 E6 and HPV18 E6 proteins; and another type of monoclonal antibody capable of binding to HPV16 L1 and HPV18 L1 proteins, among others, to be used for various immunological assays.

[0053] Still another aspect of the invention provides a monoclonal antibody capable of binding to an early HPV viral protein and a late HPV viral protein from different HPV types. The monoclonal antibody may be obtained by screening antibody-producing hybridoma cells with a purified recombinant papillomavirus early protein from one HPV type and a purified recombinant papillomavirus late protein from another HPV type, wherein the screening step includes selecting the antibody-producing hybridoma cells with positive reactivity to the purified recombinant papillomavirus early protein and the purified recombinant papillomavirus late protein, and with negative reactivity to non-HPV proteins, such that the monoclonal antibody generated from the antibody-producing hybridoma cells is capable of binding to the early HPV viral protein and the late HPV viral protein from different HPV types. The purified recombinant papillomavirus early protein may include HPV16 E6 protein, HPV16 E7 protein, HPV18 E6 protein, HPV18 E7 protein, and combinations thereof, and the purified recombinant

papillomavirus late protein may include HPV16 L1 protein, HPV18 L1 protein, and combinations thereof. Exemplary monoclonal antibodies include a type of monoclonal antibody capable of binding to all HPV16 E6, HPV16 E7, HPV16 L1, HPV18 E6, and HPV18 E7 proteins; another type of monoclonal antibody capable of binding to all HPV16 E6, HPV16 E7, HPV16 L1, HPV18 E6, HPV18 E7, and HPV18 L1 proteins, among others. Such type of monoclonal antibody produced by the method of the invention can be used to detect the presence of any of these viral proteins in one or more immunological assays.

[0054] Still another aspect of the invention provides HPV type-specific monoclonal antibody capable of binding to only a first HPV viral protein, but not a second HPV viral protein different from the first HPV viral protein. Such a monoclonalantibody may be obtained by screening antibody-producing hybridoma cells with positive reactivity to a first purified recombinant papillomavirus protein from a first HPV type and with negative reactivity to a second purified recombinant papillomavirus protein from a second HPV type, wherein the first and second viral proteins correspond to the first and the second purified recombinant papillomavirus proteins of the first and second HPV types. The HPV type-specific monoclonal antibody may be capable of binding to only one viral protein, the first viral protein. The first viral protein may include a viral protein from a HPV type, such as high risk HPV types, low risk HPV types, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68, HPV-6, HPV-11, HPV-42, HPV-43, HPV-44, HPV-53, HPV-54, HPV-55, and HPV-56, and combinations thereof. Exemplary monoclonal antibodies include monoclonal antibodies recognizing only one viral protein selected from the group of HPV 16 E6 protein, HPV 16 E7 protein, HPV 16 L1 protein, HPV 18 E6 protein, HPV18 E7 protein, and HPV 18 L1 protein. Such type of monoclonal antibody produced by the method of the invention can be used to detect the presence of a specific viral protein in one or more immunological assays.

[0055] In one embodiment, the various recombinant papillomavirus proteins are purified from soluble fractions obtained after lysis of cells expressing the

papillomavirus recombinant proteins such that they are soluble purified recombinant proteins. For example, the recombinant proteins may be purified into native folding in solution. As another example, the recombinant proteis are purified with buffers at pH values close to its pI or neutral pH or PBS buffers. As an example, the various recombinant papillomavirus proteins can be purified into its native folding by, e.g., dialysis in mild buffer solutions at neutral pH or in PBS buffer; concentrating by spin column or concentrators, *etc.*, to maintain its solubility and obtain higher concentration. One method of purifying HPV recombinant proteins are described in co-pending United State patent application serial number 11/559,366, filed on November 13, 2006, titled "Detection method for human pappilomavirus (HPV) and its application in cervical cancer", which is incorporated by reference herein.

[0056] The sources of HPV proteins for making monoclonal antibodies are not limited; they can be various proteins from various HPV genes and from various HPV types/species. HPV viral protein and/or oncoproteins of the invention include, but are not limited to, HPV E6 proteins, HPV E7 proteins, HPV L1 proteins, HPV E2 proteins, HPV E3 proteins, HPV E4 proteins, HPV E5 proteins, HPV L2 proteins, among others.

[0057] The HPV types are not limited. In general, HPV are divided into at least three groups: (1) the high risk HPV types, including α-papillomaviruses HPV-16, HPV-18, HPV-31, HPV-33, HPV-45, HPV-35, HPV-52, HPV-58, etc., which are oncogenic for cervical cancer and other cancers; (2) the low risk HPV types (including α-papillomaviruses HPV-6, HPV-11, HPV-13, HPV-34, HPV-44, HPV-55, HPV-73, HPV-27, PCPV1, HPV-2a, HPV-57, etc., which are at low risk for developing into cervical and other cancers; (3) other non-oncogenic α-papillomaviruses (such as HPV-66, HPV-68, HPV-53, HPV-51, HPV-59, HPV-30, HPV-26, HPV-10, HPV-28, HPV-32, HPV-39, HPV-3, HPV-29, HPV-70, etc.). Multiple HPV infection in a single human subject can be caused by two of more HPVs among the three HPV groups (high risk, low risk, and/or non-oncogenic).

[0058] Two problems existed such that there is no antibody available to do clinical HPV diagnostics. One is that HPV proteins in clinical sample are present in very small quantities. Secondly, the HPV types in the clinical sample are generally unknown. Therefore, people skilled in the art failed and were not able to produce large scale production of HPV antibodies that recognizes various clinical HPV types, despite this long felt need, and the need is not solved. For successful use of an antibody in a diagnostic test the antibody must recognize an epitope which is not only present on the immunogen, but also the epitope can be exposed in the test sample being prepared for analysis (i.e., exposed after any pre-treatment of tissues such as cryopreservation, sectioning and fixing). Therefore, the method chosen for screening large numbers of hybridoma culture supernatants must be such that it aids selection of diagnostically useful antibodies. There has been known failure in purifiying and obtaining recombinant HPV proteins and in producing antibodies from purified recombinant proteins for detecting HPV infection and people have not been able to solve the need. The invention provides purified recombinant HPV proteins that can be used as immunogens for producing anti-HPV antibodies and screesning antibody-producing hybridoma cells, such that the generated anti-HPV antibody can be used on clinical samples.

[0059] Methods of producing monoclonal antibobies against HPV proteins are provided herein to obtain one or more monoclonal antibodies, each monoclonal antibody is capable of recognizing a common epitope or a sepcific epitope of HPV proteins among various HPV proteins and/or HPV types. In addition, some of the monoclonal antibodies obtained herein are HPV type-specific, while some of the monoclonal antibodies obtained herein are non-HPV type-specific. The non-HPV type-specific antibodies are useful to detect prevalent HPV types present in clinical samples. As a result, these monoclonal antibodies are sutiable to be used in an assay detecting for HPV infection in one or more clinical samples. Epitope mapping of these non-HPV type specific antibodies identifies and allocates the common epitope of the HPV specific proteins for binding of these monoclonal antibodies.

[0060] In one embodiment, a monoclonal antibody capable of recognizing a common epitope on two or more HPV viral proteins is obtained by screening antibody-producing hybridoma cells with two or more purified HPV recombinant proteins. The two or more purified HPV recombinant proteins may include any of the suitable HPV proteins, such as HPV 16 E6 protein, HPV 16 E7 protein, HPV 16 L1 protein, HPV 18 E6 protein, HPV18 E7 protein, HPV 18 L1 protein, and combinations thereof.

[0061] In another embodiment, the two or more HPV proteins correspond to the two or more purified recombinant papillommavirus proteins. For example, when the two or more HPV protein are E6 and E7 early proteins, a monoclonal antibody recognizing a common epitop on both E6 and E7 early proteins can be obtained by screening two or more purified E6 and E7 recombinant proteins from HPV16 and/or HPV 18, or another HPV type. In another embodiment, the two or more purified recombinant papillomavirus proteins are two or more recombinant HPV early proteins such that the monoclonal antibody is capable of recognizing a common epitope on HPV early proteins corresponding to the two or more purified recombinant HPV early proteins.

[0062] One example is a monoclonal antibody capable of recognizing a common epitope on both HPV16 E6 and HPV16 E7 early proteins by screening antibody-producing hybridoma cells with a purified HPV16 E6 recombinant protein and a purified HPV16 E7 recombinant protein. Another example is a monoclonal antibody capable of recognizing a common epitope on both HPV18 E6 and HPV18 E7 early proteins by screening antibody-producing hybridoma cells with a purified HPV18 E6 recombinant protein and a purified HPV18 E7 recombinant protein.

[0063] In another embodiment, the two or more purified recombinant papillomavirus proteins include a purified recombinant papillomavirus early protein and a purified recombinant papillomavirus late protein such that the monoclonal antibody is capable of recognizing a common epitope on early and late viral proteins corresponding to the purified recombinant papillomavirus early protein and

the purified recombinant papillomavirus late protein. The purified recombinant papillomavirus early protein may be any of the early papillomavirus proteins from any of the papillomavirus types, such as HPV 16 E6 protein, HPV 16 E7 protein, HPV 18 E6 protein, HPV18 E7 protein, and combinations thereof. The purified recombinant papillomavirus late protein may be any of the late papillomavirus proteins, such as HPV16 L1 protein, HPV18 L1 protein, and combinations thereof. For example, a monoclonal antibody is obtained by screening with HPV16 E6, HPV16 E7, HPV16 L1, HPV18 E6, and HPV18 E7 proteins such that the monoclonal antibody recognizes a common epitope on HPV16 E6, HPV16 E7, and HPV16 L1 proteins.

[0064] In still another embodiment, the two or more purified recombinant papillomavirus proteins are two or more recombinant papillomavirus proteins from at least two different papillomavirus types. For example, a monoclonal antibody is obtained by screening antibody-producing hybridoma cells with a first purified recombinant papillomavirus protein from a first HPV type and a second purified recombinant papillomavirus protein from a second HPV type. The papillomavirus types can be any of the papillomavirus types, such as high risk HPV types, low risk HPV types, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68, HPV-6, HPV-11, HPV-42, HPV-43, HPV-44, HPV-53, HPV-54, HPV-55, and HPV-56. The first and the second purified recombinant human papillomavirus viral proteins may be, for example, HPV 16 E6 recombinant protein, HPV 16 E7 recombinant protein, HPV 16 L1 recombinant protein, HPV 18 E6 recombinant protein, HPV18 E7 recombinant protein, HPV 18 L1 recombinant protein, and combinations thereof.

[0065] Accordingly, one example is a monoclonal antibody capable of recognizing a common epitope on E6 protein from two different HPV types, both HPV16 and HPV18, by screening antibody-producing hybridoma cells with a purified HPV16 E6 recombinant protein and a purified HPV18 E6 recombinant protein. Another example is a monoclonal antibody that recognizes a common epitope on HPV16 E7 and HPV18 E7 proteins. Still another example is a

monoclonal antibody that recognizes a common epitope on HPV16 E6, HPV16 E7, HPV16 L1, HPV18 E6, and HPV18 E7 proteins.

[0066] In still another embodiment, a monoclonal antibody capable of recognizing a specific epitope on only one HPV protein, but not another HPV protein is obtained by screening antibody-producing hybridoma cells with a first purified recombinant papillomavirus protein from a first HPV type and a second purified recombinant papillomavirus protein from a second HPV type, wherein the one and another viral proteins correspond to the first and the second purified recombinant papillomavirus proteins of the first and second HPV types.

[0067] One example is a monoclonal antibody obtained by screening antibody-producing hybridoma cells with a purified recombinant HPV16 E6 protein and a purified recombinant HPV18 E6 protein, such that the monoclonal antibody recognizes a specific epitope on HPV16 E6 protein and does not recognize or interact with HPV18 E6 protein. Another example is a monoclonal antibody obtained by screening antibody-producing hybridoma cells with a purified recombinant HPV16 E6 protein and a purified recombinant HPV18 E6 protein, such that the monoclonal antibody recognizes a specific epitope on HPV18 E6 protein and does not recognize or interact with HPV16 E6 protein.

[0068] Another example is a monoclonal antibody obtained by screening antibody-producing hybridoma cells with a purified recombinant HPV16 E7 protein and a purified recombinant HPV18 E7 protein, such that the monoclonal antibody recognizes a specific epitope on HPV16 E7 protein and does not recognize or interact with HPV18 E7 protein. Another example is a monoclonal antibody obtained by screening antibody-producing hybridoma cells with a purified recombinant HPV16 E7 protein and a purified recombinant HPV18 E7 protein, such that the monoclonal antibody recognizes a specific epitope on HPV18 E7 protein and does not recognize or interact with HPV16 E7 protein.

[0069] In another embodiment, various monoclonal antibodies against HPV proteins, E6, E7 or L1 (anti-HPV E6, anti HPV E7, anti-HPV-L1) are provided including those monoclonal antibodies specific for detecting HPV types correlated with the immunogens that the antibodies were raised, and other non-HPV type-specific monoclonal antibodies. The antibodies of the invention includes, but are not limited to anti-E6, anti-E7, and anti-L1 antibodies, etc., and are used in one or more immunological assays. For examples, the monoclonal antibodies can be used to test on various biological samples, cell lines, and/or clinical samples of various grades of epithelial lesions (CIN2, CIN3, LSIL, HSIL, ASCUS) as well as different cervical cancers, squamous cell carcinoma (SCC, a type of common cancer) and adenocarcinoma (ADC, a type of gland cancer).

[0070] In one embodiment, a method of screening a human subject of papillomavirus infection includes obtaining a clinical sample from the human subject, and conducting one or more immunological assays on the clinical sample from the human subject using various HPV recombinant proteins and lab-generated antibodies specific for HPV proteins in order to detect and screen for the presence of HPV infection from the presence of HPV antibodies and HPV proteins in the human subject. In another embodiment, the HPV proteins in the human subject are detected using antibodies raised against HPV recombinant proteins, including but not limiting to various polyclonal and monoclonal antibodies against various HPV early and late proteins. .

[0071] The antibodies as developed herein lend themselves to the high quality and properly purified recombinant proteins encoded by HPV early and late genes, useful in immunological assays to generate very high sensitivity and specificity for screening HPV infection and cervical cancer detection. The monoclonal antibody can be used for one or more immunological assays selected from the group consisting of ELISA (enzyme linked imunoabsorbant sandwich assays), antigen assays for papillomavirus proteins, antibody assays for antibodies against papillomavirus proteins, assays for papillomavirus immunocomplexes, protein chip assays, radioimmunoprecipitation assays, rapid membrane

immunochromatographic assays, rapid stick immunochromatographic assays, immunohistochmistry for tissues and/or cervical cells, and immunocytological assays followed by flow cytolmetry, among others. In one embodiment, the one or more immunological assays may be non-invasive with minimal or no additional instrument required.

[0072] The basic techniques for conducting the immunological assays can be found in "Antibodies: A Laboratory Manual", Harlow and Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1989; "Molecular Cloning", A Laboratory Manual, eds. Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989, and others books and manuals known in the art. The related immunological assays, immunohistochmistry for tissues and/or cervical cells, and/or immunocytological assays followed by flow cytolmetry can also be found in co-pending United State patent applications: serial number 11/559,366, filed on November 13, 2006, titled "Detection method for human pappilomavirus (HPV) and its application in cervical cancer"; serial number 12/082,740, filed April 14, 2008, titled "Protein chips for HPV detection"; serial number 61/131,991, filed June 13, 2008 titled "Antibodies and assays for HPV detection"; serial number 61/192,912 Filed on September 22, 2008, titled "Novel monoclonal antibodies against HPV proteins useful for early stage and late stage detection, screening, and diagnosis of HPV related cervical cacner"; serial number ??/?? (NEOD/0005.01), filed concurrently as this application, titled "in situ detection of early stages and late stages HPV infection"; serial number ??/?? (NEOD/0005.02), filed concurrently as this application, titled "in situ detection of early stages and late stages HPV infection"; serial number ??/?? (NEOD/0005.03), filed concurrently as this application, titled "Detection of early statges and late stages HPV infection". All of the above referenced applications are herein incorporated by reference.

[0073] In one embodiment, the invention also provides various methods, detection assays, and kits, polyclonal and monoclonal antibodies, polypeptides, recombinant proteins, and nucleic acids useful for detecting general HPV infection as well as infection by various HPV genotypes, high risk HPVs and low risk HPVs.

In addition, the assays or sample formats in detecting the presence of HPV proteins are not limited and can be used for cervical tissues, cervical cells, cervical scrapes, serum, body fluids, etc. The useful screening or diagnosing assay can be IHC, ICC, flow cytometry, antibodies coupled to beads, rapid tests, protein chips, dot blots, slots, as well a conventional ELISA assay. HPV proteins can be detected by the antibodies of the invention to be present in epithelium tissue as evidenced by IHC staining after scoring by a pathologist.

[0074] The antibodies described in this invention provide a tool to detect HPV proteins present in various sources of biological samples. As an example, the antibodies described herein can be used as capture antibody to coat on microtiterplate and/or used as detection antibody as a sandwisch format of ELISA (Enzyme Linked Immunoabsorbent Sandwich Assay). Depending on detection of HPV proteins and/or HPV types, antibodies can be selected for use based on the specificity described herein of monoclonal antibody to particular HPV proteins or HPV types, or in combinations thereof. The detection antibody from selected specificity of monoclonal antibodies described herein can be directly conjugated with label like biotin, alkalin phosphatase, HRP, flourecent, etc., followed by color metric, chemiluminescent or fluorescent substrate for readout. The detection antibody can also select from polyclonal antibody described herein followed by a secondary antibody conjugated with label like biotin, alkalin phosphatase, HRP, flourecent, etc. Combination of using polyclonal and monoclonal antibodies for the sandwich ELISA as capture and detection antibody or vice versa, increases assay sensitivity by incoporating secondary antibody to amplify the signal for detecting the binding. For direct EIA (Enzyme Immuno Assay), cells, samples or cultured cells to be tested were collected and lysed to generate cell late as anyalyte. The protein in the cell lysate was quantitated and coated to microtiterplate using the same amount of protein for coating of each sample in each well followed by the detection antibody with specificity described herein.

[0075] Detection of HPV DNAs, genomes, early viral proteins, late viral proteins, oncoproteins, and/or capsid proteins from various HPV genotypes can be

performed by vaious in vitro and in vivo method and detection assays according to "Antibodies: A Laboratory Manual", Harlow and Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1989; "Molecular Cloning", A Laboratory Manual, eds. Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989, and others books and manuals and can be very useful in general clinical screening for HPV infection.

[0076] In addition, detection of HPV antibodies and/or oncoproteins by immunological assays can be used in general clinical screening for HPV infection and early diagnosis for cervical cancer and can be performed in a single rapid test or in multiplexed test.

[0077] Comparative detection of altered levels of HPV proteins and host proteins can be performed in the same or different immunological assays using the antibodies of the invention. It can also be used in diagnosing HPV-associated carcinomas of the uterine cervix, as well as those cases associated with epithelial cell abnormalities induced by HPV infection, pre-malignant and malignant HPV-associated epithelial cell lesions, and those at risk of developing HPV-associated cervical carcinoma and adenocarcinoma. The methods as described herein can be used independently or as an adjunct screening tool to convention cytological papanicolaou smear tests or histological tests and the results thereof can be compared for follow-up patient management.

[0078] The antibodies of the invention can be used in an immunological assay to detect a diseased stage caused by HPV infection. The disease stage may be, for example, an early stage HPV infection, a late stage HPV infection, an early stage cervical cell lesion, a late stage cervical cell lesion, low grade of squamous intraepithelial lesion (LSIL), high grade of squamous intraepithelial lesion (HSIL), atypical squamous cells of undetermined significance (ASCUS), cervical intraneoplasm stage 1, 2, 3 (CIN1, CIN2, CIN3, respectively), developed cervical cancer, adenocarcinoma (ADC) , or squamous cell carcinoma(SCC).

Examples:

1. Expression, purification, and preparation of HPV recombinant proteins to be used as immunogens for generating antiserum and anti-HPV antibodies, and screening hybridoma cell lines for monoclonal antibodies

[0079] HPV recombinant proteins can be any kinds of HPV proteins, HPV proteins of early genes and/or late genes, including, but not limited to, E2, E6, E7, L1, L2 and can be from various HPV types. Full-length E6, E7, and/or L1 polypeptide sequence, which have been found very difficult to obtain and purify due to undesirable aggregation during protein purification, protein instability, low levels of expression, low immunogenic responses of purified proteins. For example, many early E6 oncoproteins contain many cysteine amino acids and thus the correct topography of the E6 oncoproteins requires formation of many disulfide bonds properly. In addition, it was known that certain immunological assays using small peptides of early E6 and E7 proteins results in extremely low assay specificity and sensitivity and thus unsuitable as tools for clinical in vitro diagnostics. Thus, the invention provides recombinant proteins, such as recombinant hybrid proteins containing a partial sequence or a full length sequence of HPV oncogenic proteins.

[0080] 1). Cloning and production of various recombinant proteins encoded by HPV16 E6 and HPV18 E6 gene. An exemplary oncogenic E6 early gene from an exemplary HPV type, HPV-16, was clone. The HPV 16 E6 gene cloned herein is a 474 base pair (b.p.) DNA fragment containing the 157 amino acid coding region of the whole HPV-16 E6 gene and obtained by polymerase chain reaction (PCR) amplification. The DNA sequence of the isolated DNA fragment was confirmed by comparing with the sequence from Gene Bank database. Recombinant HPV-18 E6 protein was also obtained. All cloning procedures are carried out according to the protocols described in "Molecular Cloning", A Laboratory Manual, eds. Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989. In addition, HPV18 E6 gene was also cloned and the DNA sequence was confirmed.

[0081] 2). Cloning and production of various recombinant proteins encoded by HPV16 E7 and HPV18 E7 gene. An exemplary oncogenic E7 early gene from an exemplary HPV type, HPV-16, was cloned. A 294 base pair (b.p.) DNA fragment containing the 99 amino acid coding region of the entire HPV-16 E7 gene was obtained by polymerase chain reaction (PCR) amplification. The DNA sequence of the isolated DNA fragment was confirmed by comparing with the sequence from Gene Bank database. Recombinant HPV-18 E7 protein was also obtained In addition, E7 DNA fragments from different HPV types can also be cloned from different clinical samples or sources.

[0082] 3). Cloning and production of various recombinant proteins encoded by HPV16 L1 and HPV18 L1 gene. An exemplary late gene from an exemplary HPV type, HPV-16, was cloned. A 1596 base pair (b.p.) DNA fragment containing the 531 amino acid coding region of the HPV-16 L1 gene was obtained by polymerase chain reaction (PCR) amplification. The DNA sequence of the isolated DNA fragment was confirmed by comparing with the sequence from Gene Bank database. In addition, L1 DNA fragments from different HPV types can also be cloned from different clinical samples or sources.

[0083] A recombinant N-terminal fragment of HPV 16 L1 protein was also obtained from a His-tagged expression system. The molecular weight of the HPV-16 L1 N-terminal recombinant protein ais bout 34 KD. L1 C-terminal fragments can also be obtained. Recombinant HPV-18 L1 protein was also obtained and used as immunogens for generating antiserum, polyclonal and monoclonal antibodies.

[0084] The one or more recombinant proteins as described herein can be expressed in various suitable systems, such as bacterial expression systems, viral expression systems, yeast expression systems, mammalian expression systems, *e.g.*, in *E coli*, yeast, baculovirus, and/or mammalian cell cultures, generally known in the field. Although the polypeptides could be obtained by other means, embodiments of the invention provide one or more recombinant proteins mostly in (or close to) their native forms, which may be a much desirable conformation for

binding with antibodies from tissues of human subjects with HPV infection in an immunological assay. For example, GST, MBP, or His tagged-HPV16-E6, HPV18 E6, HPV16 E7, HPV18 E7, HPV16 L1, and HPV18 L1 recombinant proteins were expressed in *E.coli BL21(DE3)* using IPTG driven induction. After induction of protein expression, tagged-HPV recombinant proteins were obtained from soluble fraction after lysis of the cultured cells and purified to a final concentration of about 0.1 to 1 mg/ml or higher. The purity of the recombinant HPV proteins was estimated to be>90% based on PAGE analysis. Recombinant HPV proteins were used to detect the presence of HPV antibody on clinical samples and was also used as immunogens for producing polyclonal antiserum and monoclonal antibodies.

[0085] The cell culture containing various recombinant papillomavirus proteins in various expression vectors as described herein are then scaled up to 1 liter or 10 liter, or 100 liters or higher to obtain high quantity of soluable recombinant protein for purification. The soluble fraction after cell lysis was passed through various chromatograpgy columns with appropriate expression systems to bind to the tag expressed along with the HPV recombinant proteins. The tag-HPV recombinant proteins were then eluted from the column and concentrated down to 100 ml or 10 ml to 1 ml. The purified soluble recombinant HPV proteins were further concentrated and dailysed with buffers at neutral pH or PBS buffers to be used as immunogen to generate antiserum against the HPV proteins. The soluble recombinant HPV proteins were thus purified from soluble fractions and folded close to their native folding states as in vivo natural conditions.

[0086] Obtaining high quality purified recombinant HPV proteins is critical in generating various types of monoclonal antibodies that recognizing common epitopes or specific epitopes for detecting HPV infection. The purified recombinant HPV proteins were tested to confirm its binding to the HPV antibody from the HPV infected clinical samples. Thus, such purified recombinant HPV proteins are suitable for use as immunogen to raise antiserum and generate antibodies that can recognize natural HPV viral proteins *in vivo*.

2. anti-HPV polyclonal antibody production:

[0087] Recombinant HPV E6, E7 or L1 proteins expressed in *E coli* was purified, concentrated, and dialyzed with PBS to be used as immunogens. Immunization was followed by standard protocol. Titer of each serum obtained was tested by ELISA assays followed by periodical boosting and bleeding. Production bleed from optimal titer was collected; processed serum was used to do immunoglobulin (Ig) purification via protein A columns or affinity columns. Purified IgG was used as anti-HPV antibodies for HPV immunoassays.

[0088] Monoclonal antibodies, polyclonal antibodies, and antiserum were obtained, purified, and tested herein to be able to detect HPV infection regardless of the pathogenesis of HPV infection, cell lesions, inflammatory, or cancer disease development. Other researchers have tried to develop anti-HPV monoclonal antibodies but have failed because they failed to generate sufficient HPV proteins for monoclonal antibodies production; they failed to generate monoclonal antibodies with high specificity because the immunigens were not immunogenic; or the generated antibodies were not able to recognize native forms of HPV proteins present in clinical samples with early stage HPV infection. Some antibodies raised against mutant peptides were only able to recognize late stage cervical cancer, but are not sure whether their antibodies would recognize wild type HPV native proteins or any early stage HPV infection. In addition, late stage HPV detection is too late for disease intervention and treatment.

[0089] The clinical utility of the antibodies described herein was validated by HPV immunoassays, such as ELISA assays, immunocytochemistry assays, immunohistochemistry assays, using appropriate clinical samples. The novel monoclonal antibodies and antiserum, obtained from methods of this inventon are able to interact and bind HPV viral proteins present in clinical samples, which have been confirmed to contain early stage cell lesions such as cervical intraepithelial neoplasia (CIN) as well as late stage HPV associated cervical cancer. The monoclonal antibodies and antiserum as described herein provide powerful tools to

detect and screen HPV related pathogenesis and cervical cancer development in both early stages and late stages; thus provides an avenue to intervene disease progression and a chance to provide early treatment.

3. HPV Monoclonal antibody development:

[0090] Recombinant HPV E6, E7 or L1 proteins expressed *in E coli* was purified, concentrated, and dialyzed with PBS to be used as immunogen. Immunization of mice was followed by standard procedure. Titer of the obtained serum was tested by ELISA followed by periodical boosting and bleeding. When the titer of the serum of the mice reaches optimal, fusion of the spleen cells of the mice with tumor cells was done by standard procedure. Clones of fused cells, e.g., hybridoma cells, were further cultured.

[0091] 1). Hybridoma screening: To obtain anti-HPV antibody producing hybridoma cells with pan and speicifc binding capability to various HPV proteins as described in this invention, hybridoma clones were screened with various proteins, including, not only the original immunogens but also additional HPV proteins as positive screening, and unrelated proteins as negative screeningl. For example, two or more purified HPV recombinant proteins were used to screen against each hybridoma clone to screen and obtain monoclonal antibody-producing hybridoma cell lines and to test and understand the specificity of each antibody-producing hybridoma cell line thus obtained.

[0092] As an example of hybridoma screening, antibody-producing hybridoma cells were screened with two or more purified recombinant human papillomavirus proteins such that the monoclonal antibody is capable of reacting with the two or more purified recombinant human papillomavirus proteins. The two or more purified recombinant human papillomavirus proteins include, but are not limited to, HPV 16 E6 protein, HPV 16 E7 protein, HPV 16 L1 protein, HPV 18 E6 protein, HPV18 E7 protein, HPV 18 L1 protein, and other HPV early proteins and late proteins from various HPV types.

[0093] The antibody-producing hybridoma cells were screened with postivie reactivity to all of the two or more purified recombinant human papillomavirus proteins and negative reactivity to non-HPV proteins, including BSA, his_6 tags, GST proteins, maltose binding proteins (MBP), other tags or proteins used in recombinant protein, and other readily available non-HPV proteins. As such, the monoclonal antibodies generated form such hybridoma screening is capable of binding to all of the two or more HPV viral proteins (e.g., the HPV viral proteins presen in clinical samples), which correspond to the two or more purified recombinant human papillomavirus proteins.

[0094] One example of the two or more purified recombinant human papillomavirus proteins are HPV early proteins such that the monoclonal antibody is capable of reacting with the two or more human papillomavirus early proteins. For example, one hybridoma cell line thus screened and obtained can produce a monoclonal antibody recognizing a common epitope on both HPV16 E6 and HPV16 E7 proteins. Another hybridoma cell line thus screened and obtained can produce a monoclonal antibody recognizing a common epitope on both HPV18 E6 and HPV18 E7 proteins.

[0095] Another example of the two or more purified recombinant human papillomavirus proteins includes a purified recombinant human papillomavirus early protein and a purified recombinant human papillomavirus late protein such that the monoclonal antibody produced is capable of reacting with a common epitope on the purified recombinant human papillomavirus early protein and the purified recombinant human papillomavirus late protein. The purified recombinant human papillomavirus early protein may be HPV 16 E6 protein, HPV 16 E7 protein, HPV 18 E6 protein, HPV18 E7 protein, and other HPV recombinant early proteins, and the purified recombinant human papillomavirus late protein may be HPV 16 L1 protein, HPV 18 L1 protein, and other HPV recombinant late proteins. For examples, hybridoma cell lines thus screened and obtained can produce a monoclonal antibody recognizing a common epitope on HPV16 E6, HPV16 E7, and HPV16 L1 proteins; or a monoclonal antibody recognizing a common epitope on

HPV16 E6 and HPV18 E6 proteins; or monoclonal antibody recognizing a common epitope on HPV16 E7 and HPV18 E7 proteins; or monoclonal antibody recognizing a common epitope on HPV16 E6, HPV16 E7, HPV16 L1, HPV18 E6, and HPV18 E7 proteins. More examples are provided in the drawings of this invention.

[0096] The antibody-producing hybridoma cells were also screened with a first purified recombinant human papillomavirus protein from a first HPV type and a second purified recombinant human papillomavirus protein from a second HPV type such that the monoclonal antibody is capable of reacting with a common epitope on human papillomavirus proteins from two or more different HPV types. The first and the second HPV types can be HPV 16, HPV 18, and other HPV types. The two or more different HPV types can be, for example, high risk HPV types, low risk HPV types, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68, HPV-6, HPV-11, HPV-42, HPV-43, HPV-44, HPV-53, HPV-54, HPV-55, and HPV-56. As an example, the first and the second purified recombinant human papillomavirus proteins may be recombinant HPV 16 E6 protein, recombinant HPV 16 E7 protein, recombinant HPV 16 L1 protein, recombinant HPV 18 E6 protein, recombinant HPV18 E7 protein, and recombinant HPV 18 L1 protein.

[0097] As another example of hybridoma screening, antibody-producing hybridoma cells were screened with postivie reactivity to some of the two or more purified recombinant human papillomavirus proteins and negative reactivity to some of the two or more recombinant human papillomavirus proteins and/or non-HPV proteins. As such, the monoclonal antibodies generated form such hybridoma screening is capable of binding to some HPV viral proteins but not other HPV viral proteins.

[0098] For example, a monoclonal antibody is obtained by screening antibody-producing hybridoma cells with a first purified recombinant human papillomavirus protein from a first HPV type and a second purified recombinant human papillomavirus protein from a second HPV type such that the monoclonal antibody

is capable of reacting with a specific epitope on only one of the first and the second purified recombinant human papillomavirus proteins and not the other purified recombinant human papillomavirus protein. Specific monoclonal antibodies obtained includes a monoclonal antibody capable of binding to only HPV 16 E6 protein, but not any other HPV proteins; a monoclonal antibody capable of binding to only HPV 16 E7 protein, but not any other HPV proteins; a monoclonal antibody capable of binding to only HPV 16 L1 protein, but not any other HPV proteins; a monoclonal antibody capable of binding to only HPV 18 E6 protein, but not any other HPV proteins; and a monoclonal antibody capable of binding to only HPV 18 E7 protein, but not any other HPV proteins.

[0099] 2). Hybridoma cell line stocks: Hybridoma cell line clones with desired positive reactivity and desired negative reactivity as judged by an immunosays (e.g., ELISA, EIA and other assays) were selected and cloned down to single cell. Each single cell clone was then grown up by tissue culture. When the cell numbers reach millions of cells per ml, the cells were frozen down and kept at -80 °C or in liquid nitrogen as storage stocks.

[00100] 3). Ascites production: Each hybridoma cell line was grown in tissue culture and injected to mice for ascites production. Ascites were collected and processed for immunoglobin purification by protein G columns. Purified immunoglobin from each hybridoma cell line was isotyped and used for HPV immunoassays.

4. The Specificity of each anti-HPV Antibody:

[00101] One or more immunological assays can be used to test the specificity of the monoclonal antibodies generated by screening the hybridoma cell lines with two or more HPV recombinant proteins. EIA (Enzyme Immuno Assay) and/or Western blots were used as the assay format to test the specificity of the HPV antibodies described herein. Various purified recombinant HPV proteins, including the original screening proteins used for obtaining the anti-HPV antibodies and other

proteins not used for screening, were used to coat on the microtiter plate to test the specificity of the obtained anti-HPV antibodies on EIA. Proteins in cell lysate from cervical cancer cell lines (with or without HPV infection) were also used to test the specificity of the anti-HPV antibodies by western blot. To confirm the binding and reacitivity of the HPV antibodies with proteins from HPV infected cell lines, western blot is very useful to demonstrate specific protein bands corresponding to the proteins present in the HPV-infected cell lines. These western blots proteins bands can be compared to recombinant HPV proteins at their expected molecular weight positions on SDS-PAGE gels. Cell lysate from cervical cancer cell lines, including Hela cell line (HPV18 positive), SiHa cell line (HPV16 postive) and C33A cell line (no HPV infection) were used to demonstrate detection of HPV E6, E7, or L1 by the anti-HPV monoclonal antibodies of the invention on western blot.

[00102] Various recombinant proteins in their native forms are coated on the bottoms of a microtiter plate to perform an EIA assay and test the specificity for each of the generated antibodies produced by the method of the invention. Monoclonal antibodies capable of binding to two or more HPV viral proteins from the same HPV type are obtained. Figure 1A and Figure 1B represent two different clones of hybridoma cells, each clone being capable of producing a monoclonal antibody recognizing a common epitope on both HPV16 E6 and HPV16 E7 proteins. In Figure 1A and Figure 1B, positive reactivity on EIA is only observed on recombinant HPV16 E6 and HPV16E7, and negative or no reactivity is observed on recombinant HPV18 E6, HPV 18 E7, HPV16 L1-N-terminal, HPV 16 L1, and 6x-His proteins. These data demonstrate that this type of monoclonal antibodies to bind specifically to HPV16E6 and HPVE7, but not to neither HPV16L1, nor HPV18 E6 or HPV18E7.

[00103] Monoclonal antibodies capable of binding to an early HPV viral protein and a late HPV viral protein from the same HPV type are obtained. Figure 2A shows the specificity of a monoclonal antibody capable of binding to all of the recombinant HPV E6, HPV E7 and HPV L1 proteins on EIA, with negative reactivity to HPV18 E6, HPV 18 E7, and 6x-His proteins. These data demonstrate that this

type of monoclonal antibodies reacts strongly to native forms of HPV16 E6 and L1 proteins, and weakly to native form of HPV16 E7 protein, but non-reactive to native form of HPV18 E6 or HPV18 E7 proteins. These data indicate that this type of monoclonal antibody contains a HPV 16 type-specific common epitope to be recognized by the native forms of all of the HPV16 E6, HPV16 E7 and HPV16 L1 proteins.

[00104] Figure 2B shows the results of a Western blot analysis of a monoclonal antibody capable of reacting with recombinant HPV E6, HPV E7 and HPV L1 proteins. The recombinant protein detected by western blot using the antibody described herein demonstrate detection of HPV E6 (about 18-20 kDa) and HPV L1 (about 55 kDa) protein. The bands from each recombinant protein shown with expected molecular weight indicate the monoclonal antibody described herein reacts strongly to denatured HPV16 E6 and HPV18E6, and weakly to denatured HPV L1 proteins on western blot, thus no detectable reactivity to HPV16 E7 nor HPV18 E7. Comparing the results as shown in Figure 2A and Figure 2B, these data indicate that this anti-HPV monoclonal antibody contains a HPV common epitope to be recognized by native forms of HPV16 E6, HPV16 E7 and HPV16 L1 proteins, as well as denatured forms of HPV18 E6 recombinant proteins.

[00105] Figure 2C shows the results of a Western blot using cell lysate from various cervical cancer cell lines to react with the same monoclonal antibody as used in Figure 2B. Both cell lyates of the cervical cancer cells form each cell line and recombinant proteins in their denatured forms are compared and shown here. The specificity of this monopclonal antibody with HPV E6, HPV E7 and HPV L1 proteins are confirmed as the monoclonal antibody can also recognize the HPV viral proteins present in HPV-infected cancer cell lines, but not cell line with no HPV infection. The double bands as detected by the monoclonal antibody around the standard molecular weight marker of 17 kDa demonstrate the detection of HPV E6 protein (about 18 kDa) and HPV E7 (about 15 kDa) protein from cervical cancer cell line in HeLa (HPV18-infected) and SiHa (HPV16 infected) cell lines, but not C33A (non-HPV infection) cell line. The bands on the recombinant protein lanes

shown with expected molecular weight indicate that the monoclonal antibody reacts strongly to denatured HPV16 E6 and HPV18E6 recombinant proteins, but weakly to the denatured HPV L1 recombinant proteins on western blot, and no detectable binding to HPV16E7 or HPV18E7 recombinant proteins.

[00106] Monoclonal antibodies capable of binding to an early HPV viral protein and a late HPV viral protein from different HPV type are also obtained. Figure 3A shows the specificity of a monoclonal antibody capable of reacting with recombinant E6, E7 and L1 proteins from both HPV16 and HPV 18 types on EIA. These data demonstrate this type of monoclonal antibody reacts specifically to all of the recombinant E6, E7 and L1 proteins of HPV16, and the recombinant E6 and E7 proteins of HPV18, but not to the his-tag peptide (even though the recombinant proteins are fused to 6x-His for expression and purification). These data indicate that this antibody recognize a common epitope shared by different HPV types, HPV16 and HPV18, as evidenced by its ability to react with all of the recombiant HPV16 E6, HPV16 E7, HPV16 L1, HPV18 E6, and HPV18 E7 proteins.

[00107] Figure 3B shows the results of a Western blot using the monoclonal antibody as used in Figure 3A. Positive reactivity of this monoclonal antibody to these recombinant proteins (i.e., recombinant E6, E7 and L1 proteins of HPV16 and HPV18) demonstrate that the monoclonal antibody is capable of recognizing E6 (about 18 kDa), E7 (About 15kDa) and L1 (about 55 kDa) proteins. The resulting bands from each recombinant protein lane of the Western blot analysis showed up at the expected molecular weight position and indicated that this monoclonal antibody reacts strongly to denatured E6 and E7 proteins from both HPV 16 and HPV18, and weakly to denatured L1 proteins on western blot. The results of Figure 3A and Figure 3B indicate that this monoclonal antibody recognize a HPV common epitope and is capable of reacting with native and denatured form of HPV16 E6, HPV16 E7, HPV16 L1, HPV18 E7 and HPV18 E6 proteins.

[00108] Figure 3C shows the results of a Western blot using cell lysate from various cervical cancer cell lines to react with the same monoclonal antibody as

used in Figure 3A and 3B. Both the cell lyate and recombinant proteins in their dentured forms are tested and shown here. The double bands as detected by the monoclonal antibody around the standard molecular weight marker of 17 kDa demonstrate the detection of HPV E6 proteins (about 18 kDa) and HPV E7 (about 15 kDa) protein from cervical cancer cell line in HeLa (HPV18) and SiHa (HPV16) cell lines, but not C33A (non-HPV infection) cell line. The bands on the recombinant protein lanes shown with expected molecular weight indicate that the monoclonal antibody reacts strongly to denatured HPV16 E6, HPV18 E6, HPV18 E7 recombinant proteins, but weakly to denatured HPV L1 recombinant proteins, and no detectable binding to HPV16E7 recombinant proteins on the Western blot.

[00109] Monoclonal antibodies capable of binding to tow or more HPV viral proteins from different HPV type are also obtained. For example, a monoclonal antibody capable of reacting with recombinant E6 proteins of HPV 16 and HPV18 is obtained. Figure 4A shows the specificity of a monoclonal antibody capable of recognizing a common epitope and reacting with recombinant HPV16 E6 and HPV18E6 proteins on EIA. Recombinant proteins in their native forms are coated on the bottom of a microtiter plate. These data demonstrate the monoclonal antibody reacts strongly to native forms of recombinant HPV16 E6 and HPV18E6 proteins, but non-reactive to native forms of recombinant HPV E7 nor HPV L1 proteins. These data indicate that this antibody recognizes a HPV E6 common epitope regardless of HPV types.

[00110] Figure 4B shows the results of a Western blot using cell lysates from various cervical cancer cell lines to react with the same monoclonal antibody as used in Figure 4A, with binding specificity to HPV 16 and HPV18 E6 viral proteins present in HPV infected cancer cell lines. The single band as detected by the monoclonal antibody around the standard molecular weight marker of 17 kDa demonstrate the detection of HPV E6 protein (about 18 kDa) from cervical cancer cell line in HeLa (HPV18), but not C33A (non-HPV infection) cell line. The bands on the recombinant protein lanes shown with expected molecular weight indicate

that the monoclonal antibody reacts strongly to denatured HPV18 E6 recombinant proteins.

[00111] Another example of the type of monoclonal antibody capable of binding to one or more HPV viral proteins from different HPV is a monoclonal antibody with binding specificity to HPV16 E7 and HPV18 E7 proteins. Figure 5 shows the specificity of such a monoclonal antibody on EIA assay. These data demonstrate the monoclonal antibody described herein reacts strongly to native forms of recombinant HPV16 E7 and HPV18 E7 proteins, but non-reactive to native forms of recombinant HPV E6 nor recombinant HPV L1 proteins. These data indicate that this antibody recognize an E7-only common epitope regardless of the HPV types.

[00112] Monoclonal antibodies capable of binding to only a first HPV viral protein, but not a second HPV viral protein different from the first HPV viral protein are also obtained. Figure 6 shows the specificity of a monoclonal antibody capable of reacting with recombinant HPV16 E6, but not with other recombinant HPV proteins on EIA. This monoclonal antibody containing specific epitope capable of reacting with HPV16 E6 only, but no cross reactivity to HPV18 E6 or other recombinant HPV proteins is found. These data demonstrate the monoclonal antibody described herein reacts strongly to native form of recombinant HPV16 E6 protein, but not-reactive to native forms of recombinant HPV E7 or L1 proteins. These data also indicate that this antibody capable of reacting with HPV16 E6 protein only recognizes a HPV16 E6 specific epitope.

[00113] As an another example, Figure 7 shows the specificity of a monoclonal antibody capable of reacting with recombinant HPV18 E6 protein, but not with other recombinant HPV proteins on EIA. This monoclonal antibody has specificity for HPV18 E6 protein only with no cross reactivity to HPV16 E6 or other recombinant HPV proteins. These data indicate that this antibody capable of reacting with only HPV18 E6 protein recognizes a HPV18 E6 specific epitope.

[00114] Figure 7B shows the results of a Western blot using cell lysate from various cervical cancer cell lines to react with the same monoclonal antibody as used in Figure 7A. The single band as detected by the monoclonal antibody around the standard molecular weight marker of 17 kDa demonstrate the detection of HPV E6 protein (about 18 kDa) in HeLa (HPV18) cervical cancer cell line, but not in C33A (non-HPV infection) cell line. The bands shown on the recombinant protein lanes with expected molecular weight indicate that this monoclonal antibody also reacts strongly to denatured HPV18E6 recombinant proteins.

[00115] As another example, Figure 8 shows the specificity of a monoclonal antibody capable of reacting with recombinant HPV16 E7, but not with other recombinant HPV proteins on EIA. There was no cross reactivity with HPV18 E7 protein or other recombinant HPV proteins on EIA. These data demonstrate the monoclonal antibody described herein reacts strongly to native form of recombinant HPV16 E7 proteins, but no detectable binding to native forms of recombinant HPV E6 or L1 proteins. These data indicate that this antibody capable of reacting with only HPV16 E7 protein recognizes a HPV16 E7 specific epitope.

[00116] As an another example to demonstrate a monoclonal antibody capable of binding to only a first HPV viral protein, but not a second HPV viral protein different from the first HPV viral protein, Figure 9 shows the specificity of a monoclonal antibody capable of reacting with recombinant HPV18 E7, but not with other recombinant HPV proteins on EIA. These data demonstrate the monoclonal antibody described herein reacts strongly to native form of recombinant HPV18 E7 proteins, but not-reactive to native form of recombinant HPV E6 or HPV L1 proteins. These data indicate that this antibody capable of reacting with only HPV18 E7 protein recognizes a HPV18 E7 specific epitope.

[00117] As another example, Figure 10A and Figure 10B show the specificity of two monoclonal antibody clones capable of reacting with only recombinant HPV16 L1-N terminal protein, but not with other recombinant HPV proteins on EIA. There is no cross reactivity with with HPV16 L1 or other recombinant HPV proteins on

EIA. These data demonstrate the monoclonal antibody described herein reacts strongly to native form of recombinant HPV16 L1-N terminal proteins, but not-reactive to native forms of recombinant HPV E6 or HPV E7 proteins. These data indicate that this antibody capable of reacting with only HPV16 L1 N-terminal protein recognizes a HPV16 L1 N-terminal protein-specific epitope.

[00118] As an another example, Figure 11 shows the specificity of a monoclonal antibody capable of reacting with recombinant HPV16 L1 and HPV16 L1-N terminal proteins, but not with other recombinant HPV proteins on EIA. The monoclonal antibody recognizes a specific epitope present on HPV16 L1 and HPV16 L1 N-terminal proteins. No cross reactivity to other HPV early proteins was found. These data demonstrate the monoclonal antibody described herein reacts strongly to native form of recombinant HPV 16 L1 and HPV16 L1-N terminal proteins, but not-reactive to native form of recombinant E6 or E7 proteins from HPV 16 or HPV 18. These data indicate that this antibody capable of reacting with HPV16 L1 and HPV16 L1 N-terminal protein recognizes a HPV16 L1 N-terminal-specific epitope.

[00119] The antibodies described in this invention can be used in various immunoassays. One exampleof an immunoassay is a sandwich ELISA assay using two or more anti-HPV antibody. In performing the sandwich ELLISA assay, first of all, a first anti-HPV antibody is coated on a microtiter plate; secondly, testing samples containing HPV viral proteins or recombiannt HPV proteins to be tested are added to each wells of the microtiter plate to react with the first anti-HPV antibody for capturing the HPV proteins on the bottom surfaces of the microtiter plate. Thirdly, a second anti-HPV antibody conjugated with horse radished peroxidase (HRP) or other detection agent are added. Detection of an antibody-protein complex are usually by addition of the substrate for the detection agent, such as a TMB substrate for HPR, to report the presence of the antibody-protein complex tagged with the detection agent (thus reflecting the binding activity of the anti-HPV antibodies with the HPV proteins) by a detection instrument, e.g., an ELISA reader. The sandwich ELISA assay provides specific binding of the HPV proteins by the first and the second anti-HPV antibodies, thus identifying or

confirming the specificity of the two anti-HPV antibodies. Various antibodies described in this invention was used as the coating antibody and the detection antibody to demonstrate antibody specificity.

Table 1: Sandwich ELISA assay to detect HPV E6 protein

coating antibody	anti-HPV 16 &18 E6	anti-HPV16 &18 E6	anti-HPV 18 E6	anti-HPV 18 E6
testing protein	recombinant HPV18 E6	recombinant HPV 16 E6	recombinant HPV18 E6	recombinant HPV 16 E6
detecting antibody	anti-HPV 18 E6	anti-HPV 18 E6	anti-HPV16 &18 E6	anti-HPV 16 & 18 E6
ELISA results (OD450)	1.5	0.05	1.45	0.05

[00120] As an example, Table 1 shows the experiment design and results of an ELISA (Enzyme linked Immuno Sandwich Assay) to detect the presence of HPV 18 E6 protein (an antigen test). The results show HPV18 E6 recombinant protein can be detected in the assay when coating and detecting antibody are capable of reacting with HPV18E6, while HPV16E6 recombinant protein can't be detected although the coating antibody capable of binding to HPV16E6, but the detecting antibody is specificly reacting with HPV18E6 only. Similar results were obtained when using the specific anti-HPV18E6 as coating followed by detecting antibody capable of binding to both HPV16E6, and HPV18E6. Data demonstrate the specificity of the antibody recognizes HPV18 E6 when HPV18 E6 recombinant protein is used as the testing protein in the sandwich assay, but non-reactive to recombinant HPV16 E6 protein as the antigen of the sandwich assay. The assay format described herein can be used to detect HPV18 E6 proteins present in biological samples, including but not limited to cell lysate from cervical cancer cell lines, cervical scrape samples, tissue, body fluid, serum, etc. This specific sandwich assay provides type specific assay for HPV 18, thus excludes binding of HPV16 E6 to be detectable in such assay.

[00121] As an another example to demonstrate the antibodies described in this invention can be used to detect HPV E7 protein, Table 2 shows the result of ELISA to detect the presence of HPV18 E7 recombinant protein using a monoclonal antibody against HPV 18 E7 (recognizing HPV 18 E7) for both coating and detecting antibody. Data demonstrate that the specificity of the antibody recognizes HPV18E7 using HPV18E7 recombinant protein as the antigen of the sandwich assay, but non-reactive using HPV16E7 as the antigen of the sandwich assay. The assay format described herein can be used to detect HPV E7 proteins present in biological samples, including but not limited to cell lysate from cervical cancer cell lines, cervical scrape samples, tissue, body fluid, serum, etc. This sandwich assay provides E7 specific assay for HPV 18, thus is useful for screening of HPV infection and detecting of HPV E7 oncogenic proteins.

Table 2: Sandwich ELISA assay to detect HPV E6 protein

coating antibody	anti-HPV 18 E7	anti-HPV 18 E7
testing protein	recombinant HPV18 E7	recombinant HPV 16 E7
detecting antibody	anti-HPV18 E7	anti-HPV 18 E6
ELISA results (OD450)	1.25	0.04

5. Applications of the anti-HPV antibodies

[00122] The HPV antibodies described in this invention can be used in various immunoassays for detecting general HPV infection as well as infection by various specific HPV genotypes, high risk HPVs and low risk HPVs. The samples to be used in detecting the presence of HPV proteins are not limited and can be obtained from cervical tissues, cervical cells, cervical scrapes, serum, body fluids, etc. The immunoassays useful for screening or diagnosing cervical cancer or HPV infection can be IHC assays, ICC assays, flow cytometry assays, assays using antibodies coupled to beads, rapid tests, protein chip assays, immunoassays with dot blots, immunoassays with slots, as well a conventional ELISA assay. As a screening test, the HPV antibodies can be used to detect HPV proteins *in situ* present in

epithelium cells of cervical scrape from general population in cervical cancer screening as evidenced by ICC staining scored by certified cytologists. As a confirmaing test, the HPV antibodies can also be used to detect HPV proteins *in situ* present in epithelium tissue as evidenced by IHC staining scored by certified pathologists.

[00123] 1). The reactivity of the purified anti-HPV Antibodies with HPV Proteins found in Biological Samples. To confirm the binding activity of the HPV antibodies with HPV proteins, purified HPV recombinant proteins and/or HPV containing cell lysate from biological samples can be tested on ELISA or direct EIA. Biological samples include, not not limited to, cells from cultured cell lines or from clinical samples.

Table 3. EIA detection of E6, E7, and L1 proteins in cervical cancer cell lines.

OD	Anti-HPV16 E6, HPV18 E6 antibody	Anti-HPV18 E6 antibody	Anti-HPV18 E7 antibody	Anti-HPV16 E7 antibody	Anti-HPV16 L1 antibody
Caski ($HPV16^+$)	0.392	0.48	0.442	0.464	0.355
SiHa ($HPV16^+$)	1.165	1.314	1.162	1.202	1.115
CxCa ($HPV16^+$)	1.126	1.047	0.802	0.825	0.724
Hela ($HPV18^+$)	0.779	0.762	0.734	0.654	0.652
HEC-1A (no HPV)	0.173	0.206	0.219	0.186	0.173

[00124] As an example, as data shown on Table 3, monoclonal antibodies specific to HPV E6, HPV E7 or HPV L1 protens were able to react specifically with cell lysate from various cervical cancer cell lines in a direct EIA format while using HEC-1A as negative control. Cell lysate from cervical cancer cell lines, including Caski, Siha, Cxca, Hela, and endometrial cancer cell line like HEC-1A (non-HPV infected) were used to demonstrate detection of HPV E6, E7, or L1 by the HPV

monoclonal antibody specific to HPV E6, HPV E7, and HPV L1 respectively as shown in Table 3.

[00125] Cultured cell lines tested and described herein, include, but not limited to, cervical cancer cells such as Caski (HPV16 positive), Siha (HPV16 postive), Cxca, Hela (HPV18 positive), and endometrial cancer cell line like HEC-1A (no HPV infection). For direct EIA, cells were collected, centrifuged, washed, and lysed to generate cell late as anyalyte. The protein in the cell lysate was quantitated and coated to microtiter plate using the same amount of protein for coating of each sample in each well. The plate was blocked, and detected by each of the HPV monoclonal antibody as indicated followed by HRP conjugated anti-mouse IgG. TMB substrate was added followed by a standard reaction stopping solution. OD_{450} was taken by an ELISA plate reader.

[00126] 2). The reactivity of the purified anti-HPV Antibodies with HPV Proteins found in clinical samples. Clinical samples to be tested and described herein include, but not limited to, cells from cervical scrapes, body fluid, or serum samples Clinical specimens from cervical scrapes were also obtained for detection of HPV E6, E7 or L1 protens on EIA.

[00127] Cell lysate from various sample source including cervical scrape cells in liquid based solution, culture medium (used for HPV DNA test sample), or pap smear sample demonstrate detection of HPV E6, E7, or L1 from clinical samples on EIA format using various HPV monoclonal antibody described in this invention. To perform the direct EIA described herein, specimens were processed, centrifuged, washed, and lysed to generate cell late as anyalyte. The proteins in the cell lysate was quantitated and coated to microtiterplate with the same amount of proteins for coating in each well. The plate was blocked, and detected by each HPV monoclonal antibody followed by HRP conjugated anti-mouse IgG. TMB substrate was added followed by a stopping solution. OD 450 was taken by an ELISA plate reader.

Table 4 EIA detection of E6, E7, and L1 proteins in cervical scrapes samples.

Samples Dx	Anti-HPV18 E6 antibody	Anti-HPV16 E7 antibody	Anti HPV L1 antibody
Squamous cell carcinoma (SCC)	+++	+++	+++
Squamous cell carcinoma (SCC)	+++	+++	+++
high grade HPV DNA test positive	-	-	-
high grade HPV DNA test positive	+	+	+
high grade HPV DNA test positive	-	-	-
pap smear normal, PCR negative	-	-	-
pap smear normal, PCR negative	-	-	-
pap smear normal, PCR negative	-	-	-

[00128] Results shown in Table 4 indicate that each monoclonal antibody detects HPV E6, E7, or L1 protein respectively from SCC samples using pap smear normal (HPV neg) as neg control of the assay. For samples from high-grade HPV DNA pos, one out of three is positive on the E6, E7, and L1 by EIA. These data indicate that E6, E7, or L1 proteins from SCC lysate can be detected by EIA using the monoclonal antibodies described herein, while high-grade HPV DNA positive samples (CIN1/2) may or may not contain detectable HPV E6, E7, or L1 proteins. The high-grade HPV DNA test used in this study was hc2, the only FDA approved HPV DNA test. For those HPV DNA positive but HPV EIA negative samples, it is possible false positive of the HPV DNA assay, or positive HPV DNA detection with no expression of HPV oncogenic proteins. These data indicate that HPV EIA assay described herein provides additional clinical relevance for screening of cervical cancer.

[00129] 3). The reactivity of the purified anti-HPV Antibodies with HPV Proteins *in situ* by Immunohistochemisty (IHC): Paraffin tissues blocks sectioned into 4 microns were placed on slide and baked at 60C overnight. Deparaffin/hydrate sections were unmasked followed by standard IHC staining procedures. Purified monoclonal antibodies against HPV proteins as described in this invention were diluted to use as the primary antibody. Staining procedure is followed by secondary antibody solution, washing, followed by appropriate substrate reagent to

each section. . As soon as the sections develop, immerse slides in distilled water, counterstain sections with hematoxylin, dehydrate and mount coverslips.

[00130] As an example, various cervical tissues from various stages of CIN were prepared to perform IHC assay using rabbit polyclonal anti-HPV E7 antibodies described herein. As another examples, a number of cervical biopy samples were tested in an immunohistohistochemistry (IHC) assay concurrently as a tissue microarray format using a monoclonal antibody to detect HPV proteins from a variety of HPV types (as confirmed by HPV DNA genotyping). Using a monoclonal antibody against HPV viral proteins and/or oncoproteins, this invention provides antibodies to detect the presence of HPV L1 viral proteins and E6, E7 oncoproteins in clinical samples having either single HPV infection or multiple HPV infections. A single anti-HPV monoclonal antibody as described herein can detect single HPV infection by at least HPV-6, HPV-16, HPV-18, HPV-31, HPV-33, HPV-52, etc, which are cancer-related HPV types (either high risk HPV types or low risk HPV types). A single anti-HPV monoclonal antibody can detect HPV infection by two or more HPV types, such as the combination of HPV-16, HPV-18, HPV-52, HPV-58, HPV-44, HPV-51, HPV-39, HPV-59, etc., which include high risk, low risk, and non-oncogenic α-papillomaviruses.

[00131] As an example, the HPV antibodies described in this invention can be applied in clinical utility. The results of the IHC assay demonstrate detection of the HPV E7 protein present in situ from various stages of cervical tissues using a mouse monoclonal anti-HPV E7 antibody. As another example, the antibodies described herein were also used in ICC assay using various cervical tissues from various stages of CIN. As another examples, results of IHC staining using a mouse monoclonal anti-HPV E6 antibody demonstrate detecting the HPV E6 protein present *in situ* from various stages of CIN tissues. These results indicate that HPV E6 and HPV E7 oncoproteins over-expressed in the dysplasia cells can be specificly detected by the IHC staining using the specific HPV antibodies.

[00132] As an example, Figures 12A-12D show IHC staining of CIN tissue demonstrated by a mouse monoclonal anti-HPV E6 antibody. Results indicate expression of E6 oncoprotein can be detected early in the precancerous stage of CIN2. Solid Black arrows indicate the specific staining of E6 protein in dysplasia cells, while empty clear arrows indicate the normal cells with no stain. Highly magnified images indicate localization of the E6 proteins expressed early in the nuclear of dysplasia cells.

[00133] Figure 12A shows the representative image of the dysplasia cells of CIN2 tissues stained by immunohistocytostaining (IHC) using an anti-E6 monolonal antibody. Figure 12B shows the representative image of the adjacent normal epithelium from the dysplasia tissue of the CIN2 sample of Figure 12A. Figure 12C-12D shows the representative image of the dysplasia epithelium of two CIN3 samples stained by IHC using the same anti-E6 monolonal antibody. These data suggest the IHC staining by E6 monoclonal antibody is specific in the nuclear and cytoplasm of dysplasia cells.

[00134] As an another example, Figures 13A-13D show IHC staining of squamous cell carcinoma demonstrated by mouse monoclonal HPV E7 antibody. Results indicate expression of E7 oncoprotein can be detected in the tumor cells of SCC tissue. Solid Black arrows indicate the specific staining of E7 protein in dysplasia cells, while empty clear arrows indicate the normal cells with no stain. Highly magnified images indicate localization of the E7 proteins expressed in the cytoplasm of tumor cells, but not in the normal epithelium, or stroma cells. These data suggest the IHC staining by E7 monoclonal antibody is specific in the cytoplasm of tumor cells. Figure 13A shows the representative image of the squamocarcinoma (SCC) tissue from tissue microarray stained by IHC using an anti-E7 monoclonal antibody. Figure 13B shows the representative image of the normal epithelium (15 mm away from the tumor tissue) of the SCC subject from Figure 13A. Figure 13C shows the representative image of another SCC sample from tissue microarray stained by IHC using the same anti-E7 monoclonal

antibody. Figure 13D shows the magnified representative image of the tumor cells stained in cytoplasm from Figure 13C.

4). The reactivity of the purified anti-HPV Antibodies with HPV Proteins in situ by Immunocytochemistry (ICC):

[00135] Cervical scrapes collected by Liquid based solution were processed according to the manufacture instruction. The cell preparation was divided into two parts, one for conventional papsmear, the other one for immunostaining. Monolayer of cervical cells on slide was processed by cytospin or thin prep techniques. The cells were then fixed and stained followed by immunostaining protocol. Stained cells are visualized under microscope.

[00136] As an example, Figure 14A-14C demonstrate immunocytochemistry assay using anti-HPV antibody. Figure 14A shows the representative image of cervical cells from a CIN2 cervical scrape sample prepared by thin prep and stained by ICC using a mouse monoclonal anti-HPV E7 antibody. Figure 14B shows the representative image of cervical cells from a CIN3 cervical scrape sample prepared by thin prep and stained by ICC using a mouse monoclonal anti-E6 antibody. Figure 14C shows the representative image of cervical cells from an adenocarcinoma (ADC) cervical scrape sample prepared by thin prep and stained by ICC using the same anti-E6 antibody shown in Figure 14B.

[00137] The one or more immunological assays using antibodies and purified recombinants proteins derived from HPV early and/or late genes as obtained herein serve as reliable indicators whether HPV infection has occurred. In addition, HPV associated malignancy or pre-malignant cell transformation can be assayed. One of the most useful aspects of the invention is in diagnosing cervical carcinoma, both squamous cell carcinoma and adenocarcinoma as well as any epithelial cell abnormality associated with oncogenic HPV infection including koilocytosis; hyperkerotosis; precancerous conditions encompasssing intraepithelial neoplasias or intraepithelial lesion; high-grade dysplasias; and invasive or malignant cancers.

[00138] In high grade CIN lesions, E6 and E7 are strongly expressed in host basal epithelial cells and interfere substantially with cell cycle control of these replication competent host cells. Expression of HPV oncoproteins interfers with G1-S-Phase regulation in host cells. The HPV E6 and E7 proteins target a plethora of cellular interactions, such as the inactivation of pRB by E7 and the degradation of p53 by E6. High level of HPV E7 proteins inactivates pRB and leads to disruption of E2F-Rb binding. Usually, binding of pRB to E2F blocks E2F driven cell cycle activation. In replicating cells, E2F is regulated by phosphorylation of RB. Rb phosphorylation is normally mediated by cyclin dependent kinases (CDK4, CDK6) that are controlled by several kinase inhibitors (INKs).

[00139] As a result of the loss of Rb/E2F repression and the strong activation by free E2F, the expression of a host cell protein, p16INK4a, is strongly overexpressed. In additon, S-phase genes are continuously activated since the p16INK4a mediated repression of Cdk4/6 has no downstream effect on pRb host cell protein. Since E7-dependent E2F release is not mediated by phosphorylation of pRb, the counter-regulatory p16INK4a expression has no effect on the activated cell cycle. Under physiological conditions p16INK4a is expressed when cells undergo a genomic stress situation such as substantial shortening of telomeres in ageing tissues. Also, apoptosis is abrogated by HPV E6 mediated degradation of p53. The overexpression of the cyclin dependent kinase (CDK) inhibitor, p16INK4a, is a direct consequence of deregulated HPV oncogene expression.

[00140] In addition, host cell proteins important for prolifiration and host cell genome replication may be overexpressed as a result of HPV infection. These host cell proteins include, ki67 (MIB-1), MYC cellular oncogene, Cyclin proteins (e.g., cyclin A, B, E, *etc.*), CDKN2A/p16INK4a, telomerase (e.g., TERC), replication complex proteins (e.g., MCM5, CDC6, topoisomerase II alpha (TOP2A), MCM2, minchromosome maintenance proteins 2, 4, and 5, *etc.*).

[00141] The one or more immunological assays as provided herein aims to employ user friendly procedures with simple instrument or no additional instrument

to perform in a short period of time. Comparison of the results of the various immunological assays, nucleic acid hybridization assays with cytological and histological data for the human subjects as well as demographic information serve to validate the correlation and accuracy in diagnosing HPV infection and/or cervical cancer. .

[00142] Another example of a method of screening a human subject infected with a human papillomavirus may include obtaining a clinical sample from the human subject, conducting a nucleic acid hybridization assay on the clinical sample, detecting the presence of a papillomavirus genome in the clinical sample from the human subject, conducting one or more immunological assays on the clinical sample, detecting the presence of an antibody to an early papillomavirus viral protein or the presence of the early papillomavirus viral protein in the clinical sample using a first recombinant protein of the early papillomavirus viral protein, and detecting the presence of an antibody to a late papillomavirus viral protein or the presence of the papillomavirus late viral protein in the clinical sample using a second recombinant protein of the late papillomavirus viral protein.

[00143] The one or more diagnostic immunological assays as described therein may also include obtaining polyclonal antibodies, monoclonal antibodies, and/or antiserum specific against the one or more recombinant proteins as obtained and described herein, taking a clinical sample likely to contain HPV associated proteins and/or antigens, reacting it with the obtained polyclonal antibodies, monoclonal antibodies, and/or antiserum specific for the one or more recombinant proteins, and assaying for the presence of any antibody-antigen complexes by suitable detection systems. Suitable detection system may employ various colormetric, chemilumenescent, flourescent substrates, *etc.*, specific for a secondary antibody used in each immunological assay.

[00144] Early diagnosis of HPV infection is important for successful prevention and treatment of cervical cancer. Strategies to prevent cervical cancer requires improved HPV testing/screening to cover a broad range of the worldwide

population in addition to closely follow-up those subjects with past or present HPV infection and/or pre-cancerous lesions. Importantly, it is known that infection in women for 12-15 years with HPV is required before invasive cancer to develop. It is thus important to be able to assay biomarkers for HPV infection as described herein to pre-screen women early, such that it will be possible to treat HPV infection early and prevent cervical cancer development, rather than having to rely on chemotherapy or radiation to treat cancer malignancy.

Attorney Docket No.: NEOD/0004 PCT

What is Claimed:

1. A method of producing a monoclonal antibody with binding specificity to two or more human papillomavirus viral proteins, comprising:

screening antibody-producing hybridoma cells with two or more purified recombinant papillomavirus proteins, wherein the screening step includes selecting the antibody-producing hybridoma cells with positive reactivity to the two or more purified recombinant papillommavirus proteins and with negative reactivity to non-HPV proteins, such that the antibody-producing hybridoma cells generate the monoclonal antibody with binding specificity to the two or more human papillomavirus viral proteins.

2. The method of claim 1, wherein the two or more purified recombinant papillomavirus proteins are selected from the group consisting of HPV 16 E6 protein, HPV 16 E7 protein, HPV 16 L1 protein, HPV 18 E6 protein, HPV18 E7 protein, HPV 18 L1 protein, and combinations thereof.

3. A monoclonal antibody capable of binding to two or more HPV viral proteins from different HPV types and obtained by screening antibody-producing hybridoma cells with two or more purified recombinant papillomavirus proteins, wherein the screening step includes selecting the antibody-producing hybridoma cells with positive reactivity to the two or more purified recombinant papillommavirus proteins from different HPV types and with negative reactivity to non-HPV proteins, such that the antibody-producing hybridoma cells generate the monoclonal antibody with binding specificity to the two or more HPV viral proteins.

4. The monoclonal antibody of claim 3, wherein the monoclonal antibody is capable of binding to HPV16 E7 and HPV18 E7 proteins.

5. The monoclonal antibody of claim 3, wherein the monoclonal antibody is capable of binding to HPV16 E6 and HPV18 E6 proteins.

6. The monoclonal antibody of claim 3, wherein the monoclonal antibody is capable of binding to HPV16 L1 and HPV18 L1 proteins.

7. The monoclonal antibody of claim 3, wherein the monoclonal antibody is capable of binding to an early HPV viral protein and a late HPV viral protein from different HPV types and obtained by screening the antibody-producing hybridoma cells with a purified recombinant papillomavirus early protein from one HPV type and a purified recombinant papillomavirus late protein from another HPV type, selecting the antibody-producing hybridoma cells with positive reactivity to the purified recombinant papillomavirus early protein and the purified recombinant papillomavirus late protein, and with negative reactivity to non-HPV proteins.

8. The monoclonal antibody of claim 7, wherein the monoclonal antibody is capable of binding to all HPV16 E6, HPV16 E7, HPV16 L1, HPV18 E6, and HPV18 E7 proteins.

9. The monoclonal antibody of claim 7, wherein the monoclonal antibody is capable of binding to all HPV16 E6, HPV16 E7, HPV16 L1, HPV18 E6, HPV18 E7, and HPV18 L1 proteins.

10. The monoclonal antibody of claim 3, wherein the monoclonal antibody can be used for one or more immunological assays selected from the group consisting of ELISA (enzyme linked immunoabsorbant assays), antigen assays for papillomavirus proteins, antibody assays for antibodies against papillomavirus proteins, assays for papillomavirus immunocomplexes, protein chip assays, radioimmunoprecipitation assays, rapid membrane immunochromatographic assays, rapid stick immunochromatographic assays, immunohistochemistry for tissues and/or cervical cells, and immunocytochemistry assays followed by flow cytometry, and combinations thereof.

11. A monoclonal antibody capable of binding to two or more HPV viral proteins from the same HPV type.

12. The monoclonal antibody of claim 11, wherein the monoclonal antibody is obtained by screening antibody-producing hybridoma cells with two or more purified recombinant papillomavirus proteins, wherein the screening step includes selecting the antibody-producing hybridoma cells with positive reactivity to the two or more purified recombinant papillommavirus proteins and with negative reactivity to non-HPV proteins, such that the antibody-producing hybridoma cells generate the monoclonal antibody with binding specificity to the two or more HPV viral proteins.

13. The monoclonal antibody of claim 12, wherein the two or more purified recombinant papillomavirus proteins are from the same HPV type.

14. The monoclonal antibody of claim 12 wherein the two or more purified recombinant papillomavirus proteins are from different HPV types.

15. The monoclonal antibody of claim 12, wherein each one of the two or more purified recombinant papillomavirus proteins is selected from the group consisting of HPV 16 E6 protein, HPV 16 E7 protein, HPV 16 L1 protein, HPV 18 E6 protein, HPV18 E7 protein, HPV 18 L1 protein, and combinations thereof.

16. The monoclonal antibody of claim 12, wherein the two or more purified recombinant papillomavirus viral proteins are two or more purified recombinant papillomavirus early proteins such that the monoclonal antibody is capable of binding to two or more HPV early proteins corresponding to the two or more purified recombinant papillomavirus early proteins.

17. The monoclonal antibody of claim 12, wherein the two or more purified recombinant papillomavirus proteins comprises a purified recombinant papillomavirus early protein and a purified recombinant papillomavirus late protein such that the monoclonal antibody is capable of binding to an early viral protein and a late viral proteins corresponding to the purified recombinant papillomavirus early protein and the purified recombinant papillomavirus late protein.

18. The monoclonal antibody of claim 11, wherein the monoclonal antibody is capable of binding to both HPV16 E6 and HPV16 E7 viral proteins.

19. The monoclonal antibody of claim 11, wherein the monoclonal antibody is capable of binding to all HPV16 E6, HPV16 E7, and HPV16 L1 viral proteins.

20. The monoclonal antibody of claim 11, wherein the monoclonal antibody is capable of binding to both HPV18 E6 and HPV18 E7 viral proteins.

21. The monoclonal antibody of claim 11, wherein the monoclonal antibody is capable of binding to the two or more HPV viral proteins from the same HPV type selected from the group consisting of high risk HPV types, low risk HPV types, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68, HPV-6, HPV-11, HPV-42, HPV-43, HPV-44, HPV-53, HPV-54, HPV-55, and HPV-56, and combinations thereof.

22. The monoclonal antibody of claim 11, wherein the monoclonal antibody can be used for one or more immunological assays selected from the group consisting of ELISA (enzyme linked immunoabsorbant assays), antigen assays for papillomavirus proteins, antibody assays for antibodies against papillomavirus proteins, assays for papillomavirus immunocomplexes, protein chip assays, radioimmunoprecipitation assays, rapid membrane immunochromatographic assays, rapid stick immunochromatographic assays, immunohistochemistry for tissues and/or cervical cells, and immunocytochemistry assays followed by flow cytometry, and combinations thereof.

23. A monoclonal antibody capable of binding to only a first HPV viral protein, but not a second HPV viral protein different from the first HPV viral protein, and obtained by screening antibody-producing hybridoma cells with positive reactivity to a first purified recombinant papillomavirus protein from a first HPV type and with negative reactivity to a second purified recombinant papillomavirus protein from a second HPV type, wherein the first and second viral proteins correspond to the first

and the second purified recombinant papillomavirus proteins of the first and second HPV types.

24. The monoclonal antibody of claim 23, wherein the first viral protein is selected from the group consisting of HPV 16 E6 protein, HPV 16 E7 protein, HPV 16 L1 protein, HPV 18 E6 protein, HPV18 E7 protein, HPV 18 L1 protein, and combinations thereof.

25. The monoclonal antibody of claim 23, wherein the monoclonal antibody is capable of binding to the first HPV viral protein from a HPV type selected from the group consisting of high risk HPV types, low risk HPV types, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68, HPV-6, HPV-11, HPV-42, HPV-43, HPV-44, HPV-53, HPV-54, HPV-55, and HPV-56, and combinations thereof.

26. The monoclonal antibody of claim 23, wherein the monoclonal antibody can be used for one or more immunological assays selected from the group consisting of ELISA (enzyme linked immunoabsorbant assays), antigen assays for papillomavirus proteins, antibody assays for antibodies against papillomavirus proteins, assays for papillomavirus immunocomplexes, protein chip assays, radioimmunoprecipitation assays, rapid membrane immunochromatographic assays, rapid stick immunochromatographic assay, immunohistochemistry for tissues and/or cervical cells, and immunocytochemistry assays followed by flow cytometry, and combinations thereof.

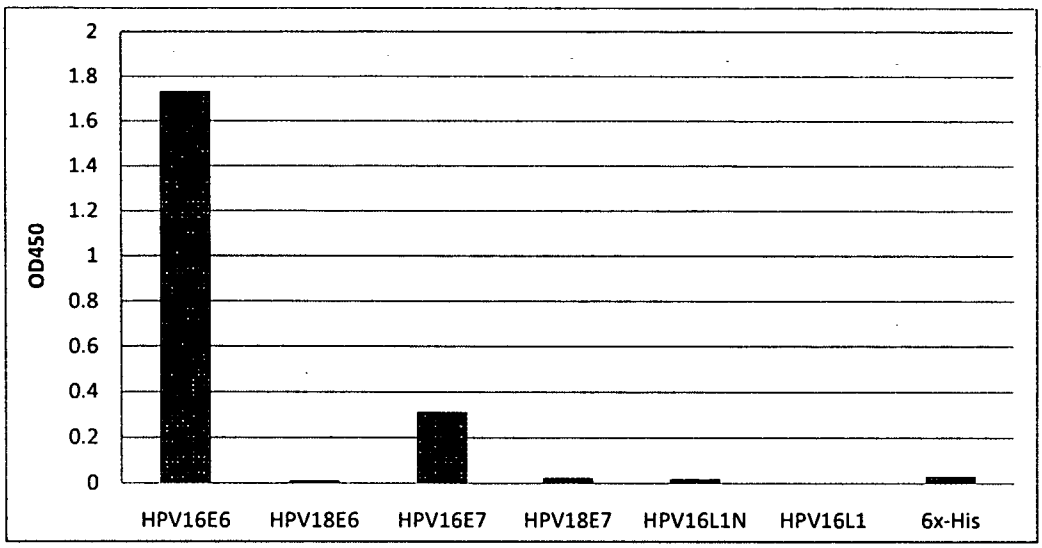

Figure 1A

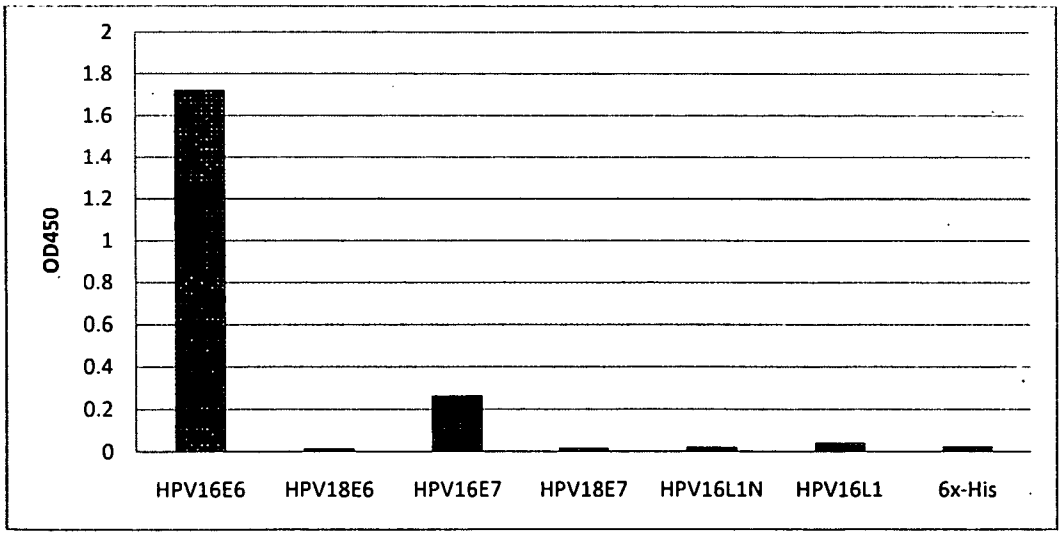

Figure 1B

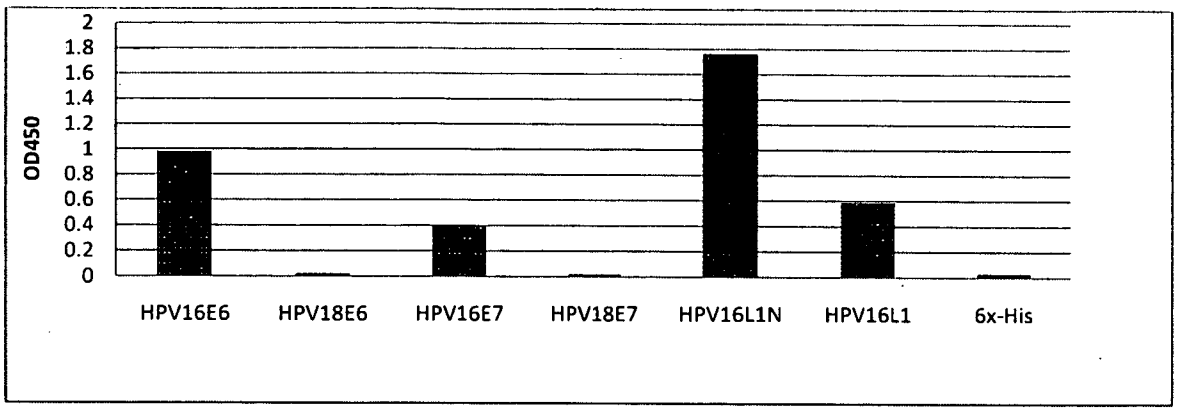

Figure 2A

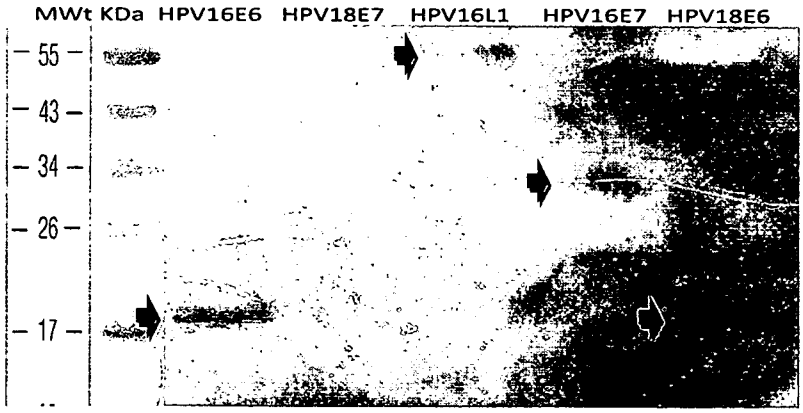

Figure 2B

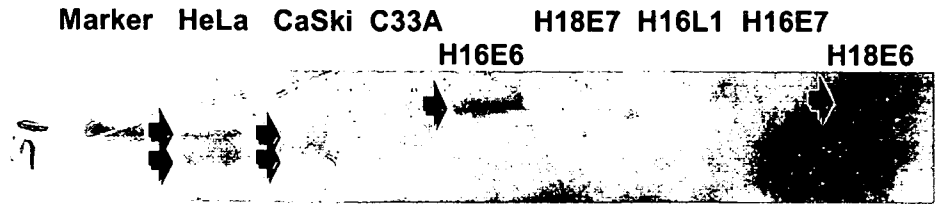

Figure 2C

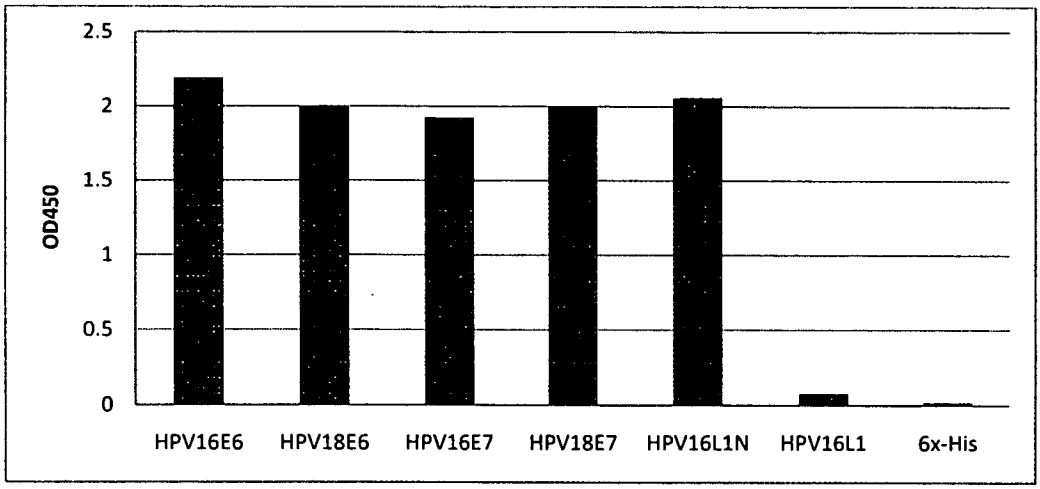

Figure 3A

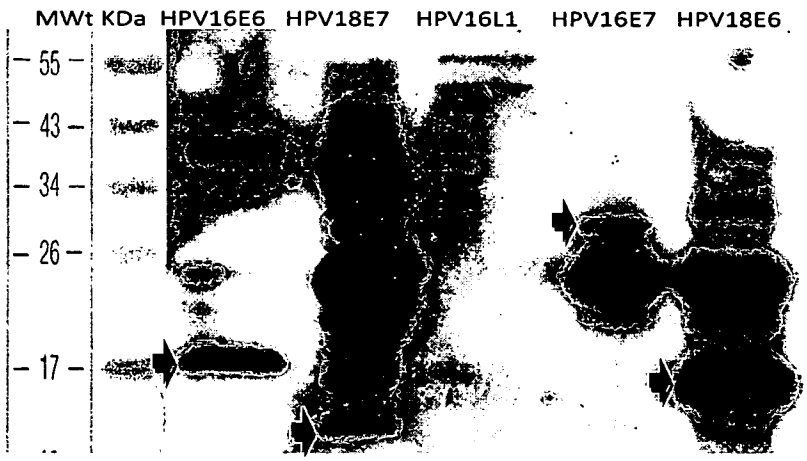

Figure 3B

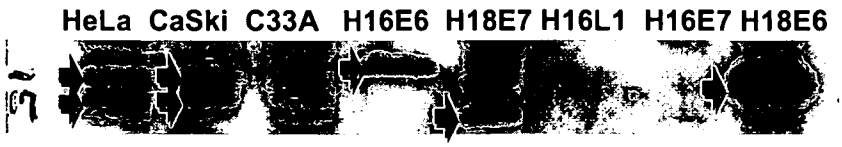

Figure 3C

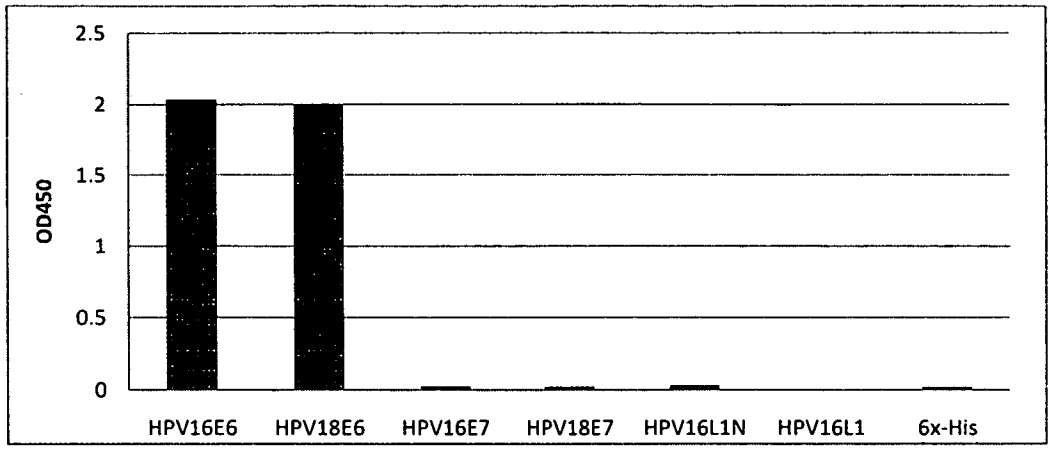

Figure 4A

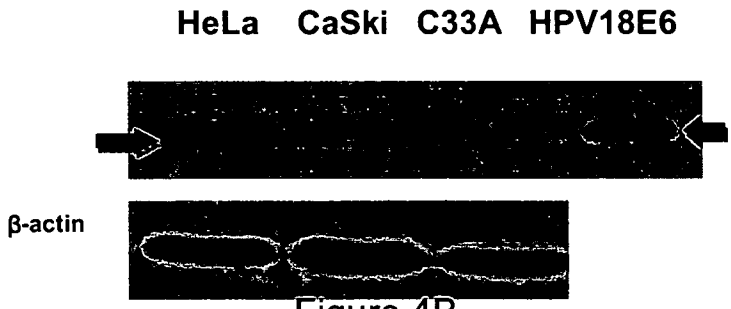

Figure 4B

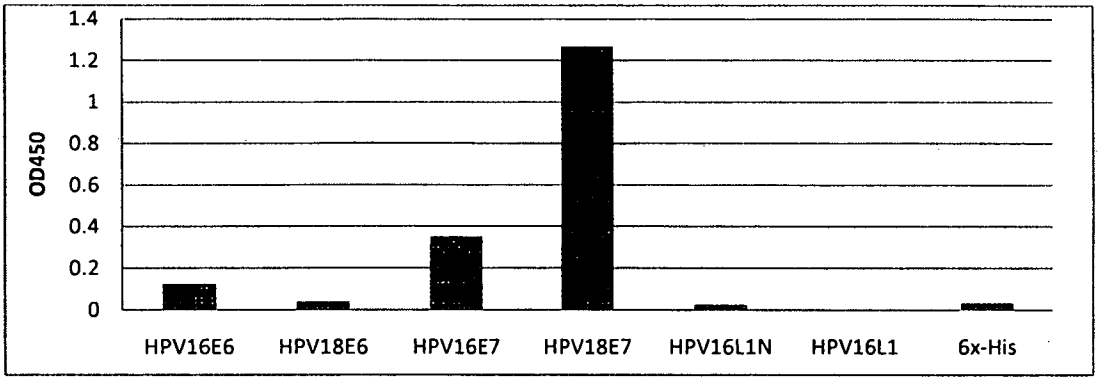

Figure 5

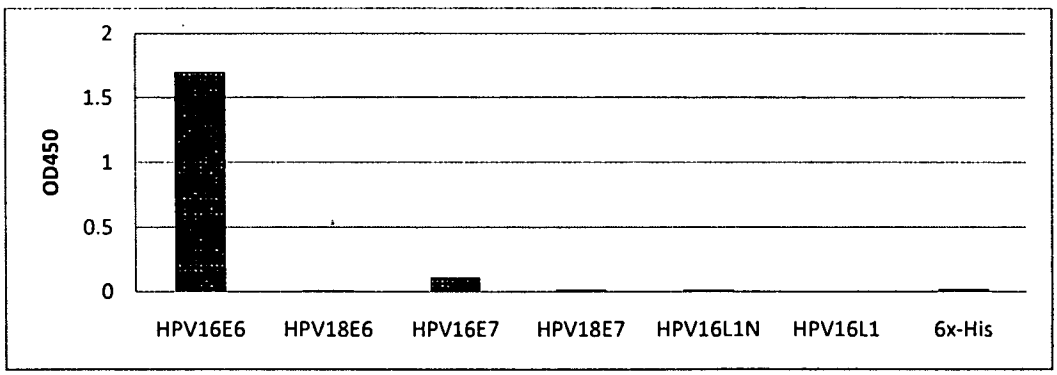

Figure 6

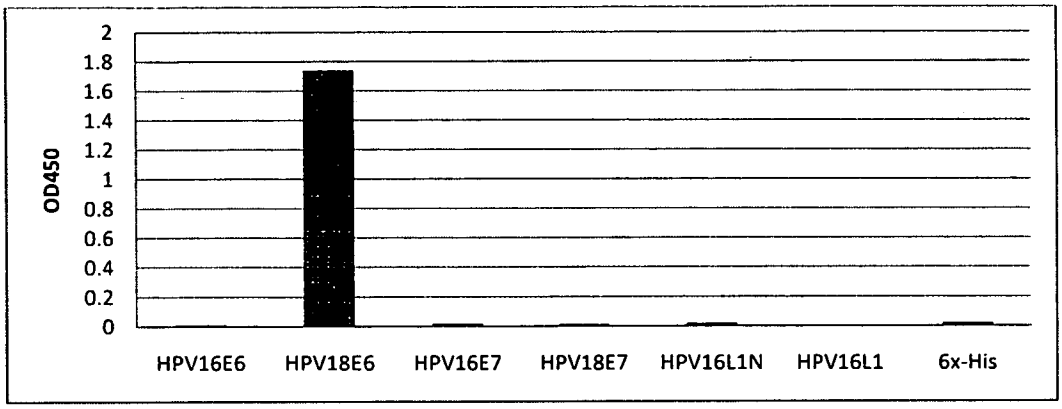

Figure 7A

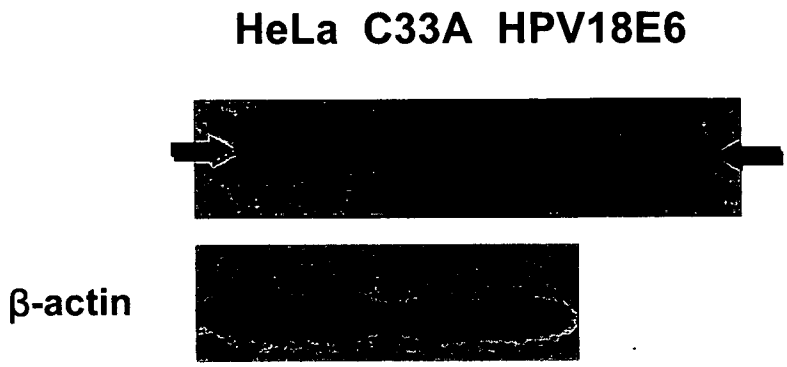

Figure 7B

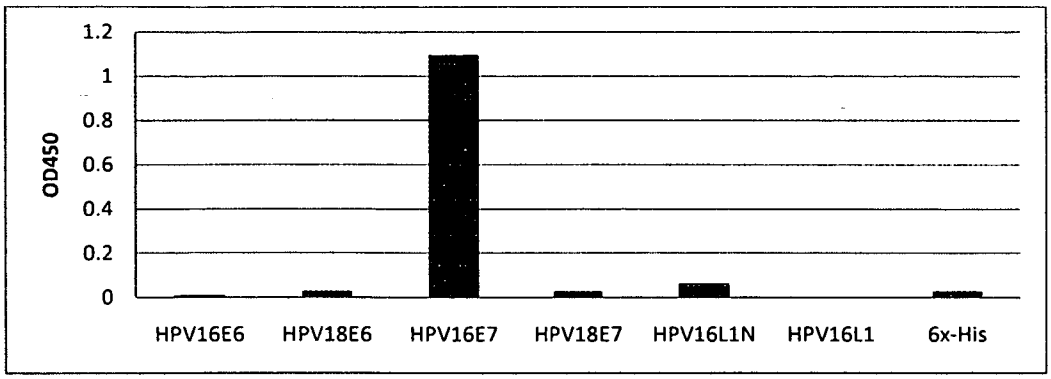

Figure 8

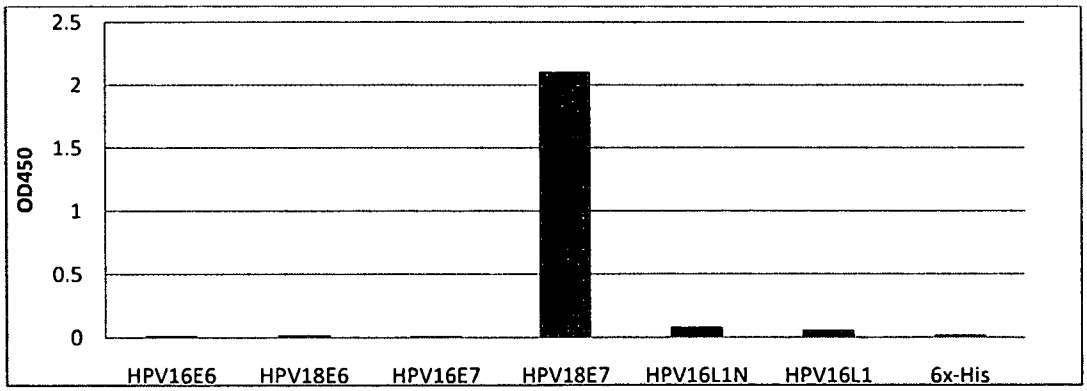

Figure 9

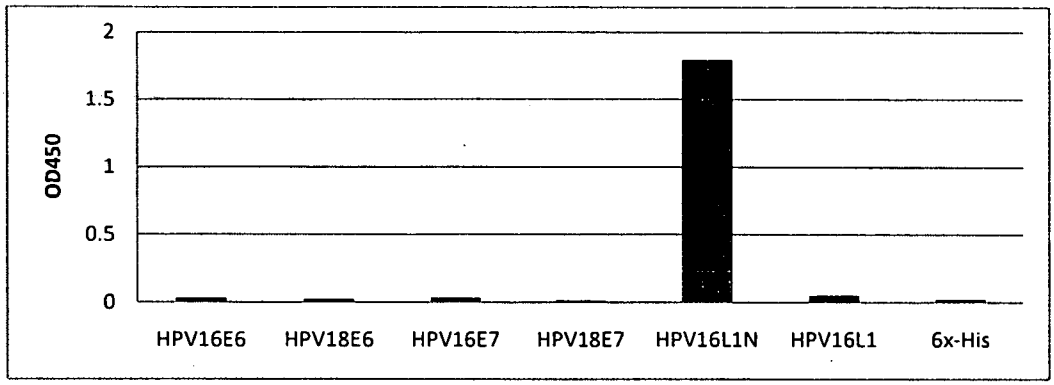

Figure 10A

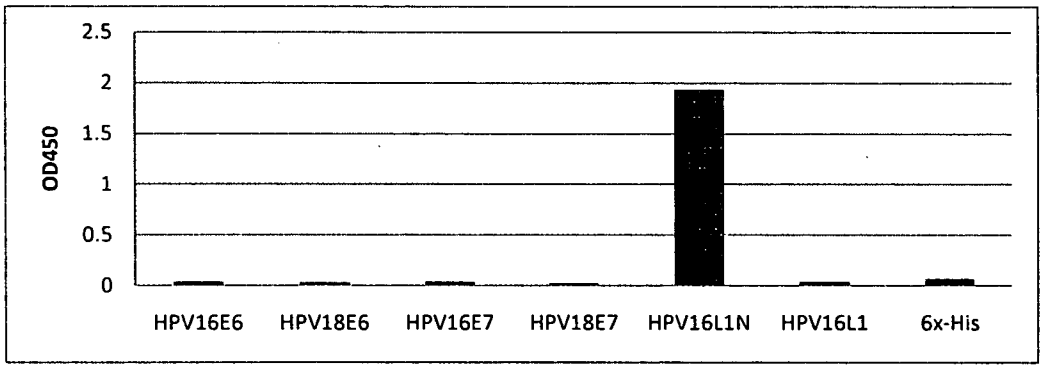

Figure 10B

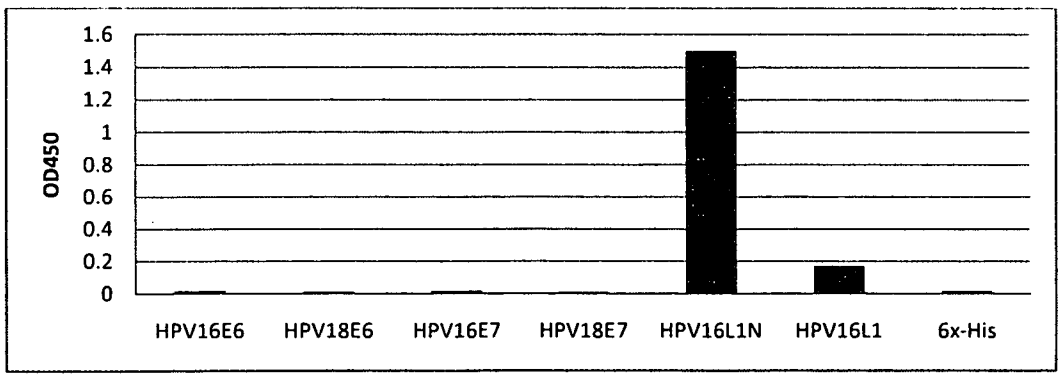

Figure 11

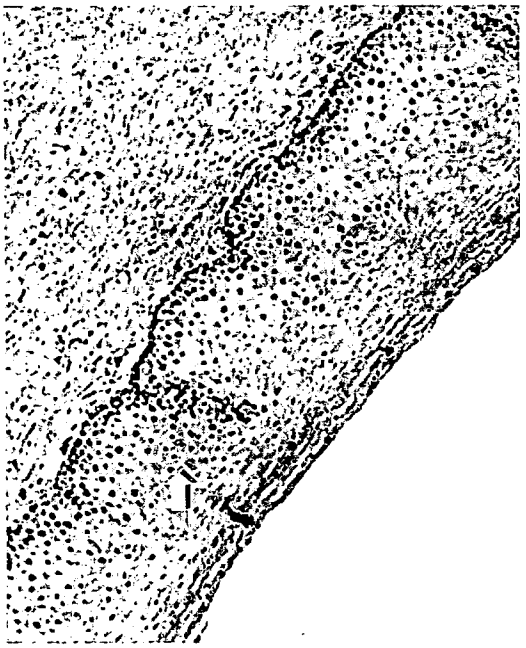

Figure 12B

Figure 12D

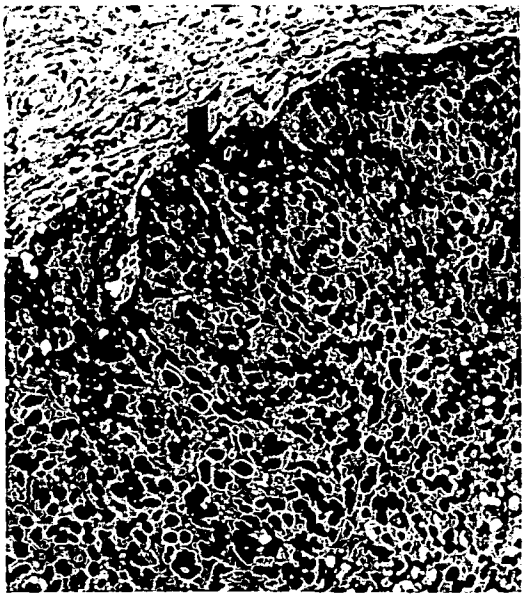

Figure 12A

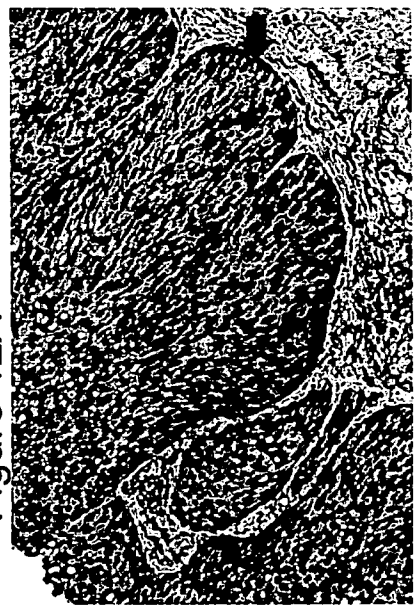

Figure 12C

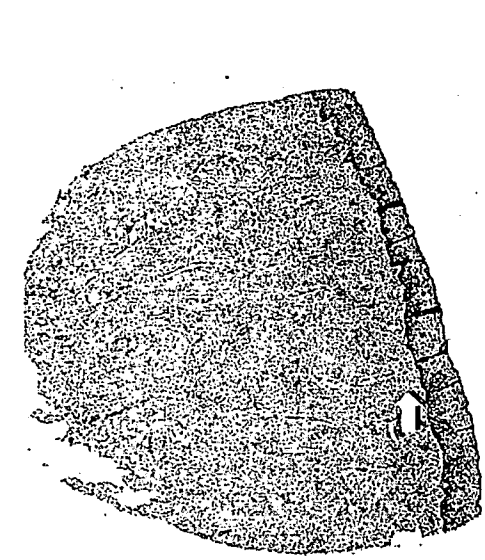

Figure 13B

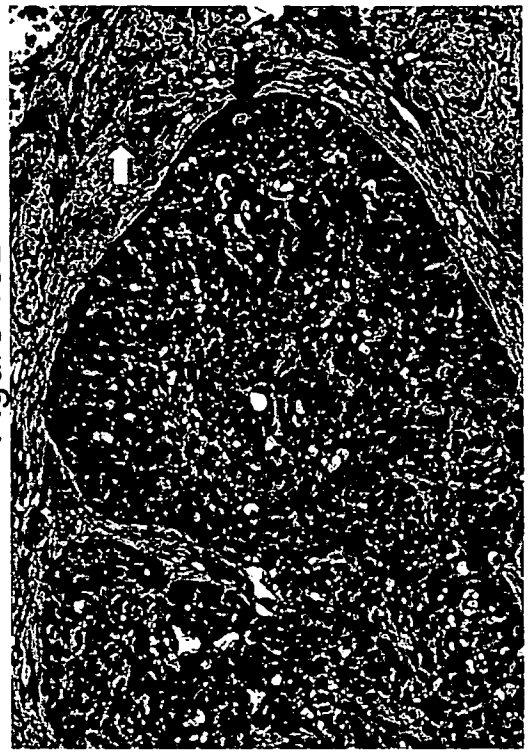

Figure 13D

Figure 13A

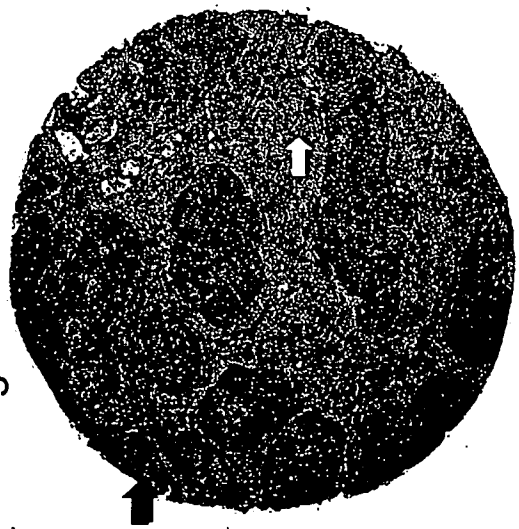

Figure 13C

Figure 14C

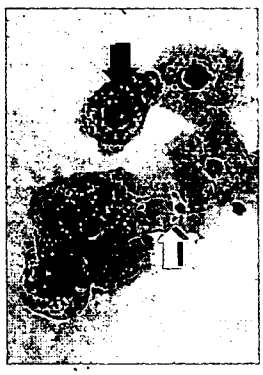

Figure 14B

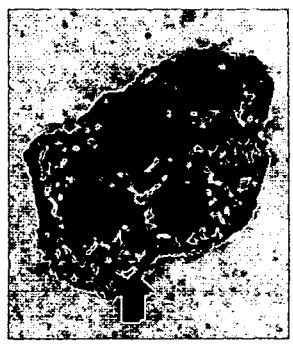

Figure 14A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/03538

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/567; C07K 16/00;C07K 17/00 (2009.01)
USPC - 435/7.21; 530/388.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): G01N 33/567; C07K 16/00;C07K 17/00 (2009.01)
USPC: 435/7.21; 530/388.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
435/70.21; 424/147.1; 935/90, 935/100, 935/104, 935/106, 935/108

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(USPT,PGPB,EPAB,JPAB); DialogPRO--Chemical Engineering and Biotechnology Abstracts, INSPEC,NTIS (National Technical Information Service), PASCAL, Current Contents Search, MEDLINE
Search Terms: HPV, monoclonal antibody, HPV 16, HPV 18, E6, E7, L1, late protein, hybridoma, ELISA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2008/0044809 A1 (Cheng) 21 February 2008 (21.02.2008), para [0034], [0035], [0042], [0043], [0060], [0061], [0120], [0140], [0182].	1-22
A	US 2007/0099199 A1 (Lu, et al.) 03 May 2007 (03.05.2007).	1-22
A	WO 2006/083984 A1 (Schiller, et al.) 10 August 2006 (10.08.2006)	1-22
A	US 2005/0159386 A1 (Kieny, et al.) 21 July 2005 (21.07.2005)	1-22
A	WO 2007/095320 A2 (Yusibov, et al.) 23 August 2007 (23.08.2007)	1-22

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September (27.09.2009)	15 OCT 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/03538

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.:
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:
This application contains the following inventions or groups of inventions which are not so linked as to from a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-22 are drawn to a method of producing a monoclonal antibody with binding specificity to two or more human papillomavirus viral proteins or a monoclonal with binding specificity to two or more human papillomavirus viral proteins.

Group II: Claims 23-26 are drawn to a monoclonal antibody capable of binding to only a first HPV viral protein, but not a second HPV viral protein different from the first HPV viral protein.
**********continued on extra sheet**********

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.:
1-22

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.

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

The groups listed above do not relate to a single general inventive concept under PCT Rule 13.1 because under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

The technical feature that links the listed Groups is a monoclonal antibody capable of binding to only a first HPV viral protein However, this does not represent an improvement over the prior art of Ohmoto et al (US 5,183,755 A) who teach such a monoclonal antibody (abstract).

Accordingly, unity of invention is lacking under PCT Rule 13 because the groups do not share a same or corresponding special technical feature providing a contribution over the prior art.