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(54) Title: MONOCLONAL ANTIBODIES AND METHODS FOR THEIR USE IN THE DETECTION OF CERVICAL DISEASE

(57) Abstract: The invention provides a novel class of compounds, pharmaceutical compositions comprising such compounds and methods of using such compounds to treat or prevent diseases or disorders associated with abnormal or deregulated kinase activity, particularly diseases or disorders that involve abnormal activation of Aik, AbI, BRK, BIK, BMX, CSK, c-Src, c-Raf, EGFR, Fes, FGFR3, Fms, Fyn, IGF-IR, IR, IKK α , IKK β , JAK2, JAK3, KDR, Lck, Met, p70S6k, Ros, Rsk1, SAPK2 α , SAPK2 β , SAPK3, SIK, Tie2, TrkB and/or WNK3 kinases.

MONOCLONAL ANTIBODIES AND METHODS FOR THEIR USE IN THE
DETECTION OF CERVICAL DISEASE

FIELD OF THE INVENTION

5 The invention relates to antibodies capable of binding to MCM2 and methods of using these antibodies, particularly in the diagnosis of cervical disease.

BACKGROUND OF THE INVENTION

10 Carcinoma of the cervix is the second most common neoplasm in women, accounting for approximately 12% of all female cancers and causing approximately 250,000 deaths per year. Baldwin *et al.* (2003) *Nature Reviews Cancer* 3:1-10. In many developing countries where mass screening programs are not available, the clinical problem is more serious. Cervical cancer in these countries is the number one cause of cancer deaths in women.

15 The majority of cases of cervical cancer represent squamous cell carcinoma, although adenocarcinoma is also seen. Cervical cancer can be prevented by population screening as it evolves through well-defined noninvasive intraepithelial stages, which can be distinguished morphologically. Williams *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:14932-14937. While it is not understood how normal cells
20 become transformed, the concept of a continuous spectrum of histopathological change from normal, stratified epithelium through cervical intraepithelial neoplasia (CIN) to invasive cancer has been widely accepted for years. The precursor to cervical cancer is dysplasia, also known in the art as CIN or squamous intraepithelial lesions (SIL). Squamous intraepithelial abnormalities may be classified by using the
25 three-tiered (CIN) or two-tiered (Bethesda) system. Under the Bethesda system, low-grade squamous intraepithelial lesions (LSIL), corresponding to CIN I and HPV infection, generally represent productive HPV infections with a relatively low risk of progression to invasive disease. High-grade squamous intraepithelial lesions (HSIL), corresponding to CIN II and CIN III in the three-tiered system, show a higher risk of
30 progression to cervical cancer than do LSIL, although both LSIL and HSIL are viewed as potential precursors of malignancy. Patient samples may also be classified as ASCUS (atypical squamous cells of unknown significance) or AGUS (atypical glandular cells of unknown significance) under this system.

A strong association of cervical cancer and infection by high-risk types of human papilloma virus (HPV), such as types 16, 18, and 31, has been established. In fact, a large body of epidemiological and molecular biological evidence has established HPV infection as a causative factor in cervical cancer. Moreover, HPV is
5 found in 85% or more of the cases of high-grade cervical disease. However, HPV infection is very common, possibly occurring in 5-15% of women over the age of 30, but few HPV-positive women will ever develop high-grade cervical disease or cancer. The presence of HPV alone is indicative only of infection, not of high-grade cervical disease, and, therefore, testing for HPV infection alone results in many false positives.
10 See, for example, Wright *et al.* (2004) *Obstet. Gynecol.* 103:304-309.

Current literature suggests that HPV infects the basal stem cells within the underlying tissue of the uterine-cervix. Differentiation of the stem cells into mature keratinocytes, with resulting migration of the cells to the stratified cervical epithelium, is associated with HPV viral replication and re-infection of cells. During
15 this viral replication process, a number of cellular changes occur that include cell-cycle de-regulation, active proliferation, DNA replication, transcriptional activation and genomic instability (Crum (2000) *Modern Pathology* 13:243-251; Middleton *et al.* (2003) *J. Virol.* 77:10186-10201; Pett *et al.* (2004) *Cancer Res.* 64:1359-1368).

Most HPV infections are transient in nature, with the viral infection resolving
20 itself within a 12-month period. For those individuals who develop persistent infections with one or more oncogenic subtypes of HPV, there is a risk for the development of neoplasia in comparison to patients without an HPV infection. Given the importance of HPV in the development of cervical neoplasia, the clinical detection of HPV has become an important diagnostic tool in the identification of patients at
25 risk for cervical neoplasia development. The clinical utility of HPV-based screening for cervical disease is in its negative predictive value. An HPV negative result in combination with a history of normal Pap smears is an excellent indicator of a disease-free condition and a low risk of cervical neoplasia development during the subsequent 1-3 years. However, a positive HPV result is not diagnostic of cervical
30 disease; rather it is an indication of infection. Although the majority of HPV infections is transient and will spontaneously clear within a 12-month period, a persistent infection with a high-risk HPV viral subtype indicates a higher risk for the development of cervical neoplasia. To supplement HPV testing, the identification of

molecular markers associated with cervical neoplasia is expected to improve the clinical specificity for cervical disease diagnosis.

Cytological examination of Papanicolaou-stained cervical smears (Pap smears) currently is the method of choice for detecting cervical cancer. The Pap test
5 is a subjective method that has remained substantially unchanged for 60 years. There are several concerns, however, regarding its performance. The reported sensitivity of a single Pap test (the proportion of disease positives that are test-positive) is low and shows wide variation (30-87%). The specificity of a single Pap test (the proportion of disease negatives that are test-negative) might be as low as 86% in a screening
10 population and considerably lower in the ASCUS PLUS population for the determination of underlying high-grade disease. See, Baldwin *et al.*, *supra*. A significant percentage of Pap smears characterized as LSIL or CIN I are actually positive for high-grade lesions. Furthermore, up to 10% of Pap smears are classified as ASCUS (atypical squamous cells of undetermined significance), i.e., it is not
15 possible to make a clear categorization as normal, moderate or severe lesion, or tumor. However, experience shows that up to 10% of this ASCUS population has high-grade lesions, which are consequently overlooked. See, for example, Manos *et al.* (1999) *JAMA* 281:1605-1610. Therefore, molecular biomarkers that are selectively overexpressed in high-grade cervical disease and compositions for the
20 detection of these biomarkers are needed to practice reliable methods for diagnosing high-grade cervical disease.

Minichromosome maintenance (MCM) proteins play an essential part in eukaryotic DNA replication. The minichromosome maintenance (MCM) proteins function in the early stages of DNA replication through loading of the prereplication
25 complex onto DNA and functioning as a helicase to help unwind the duplex DNA during de novo synthesis of the duplicate DNA strand. Each of the MCM proteins has DNA-dependent ATPase motifs in their highly conserved central domain. Levels of MCM proteins generally increase in a variable manner as normal cells progress from G₀ into the G₁/S phase of the cell cycle. In the G₀ phase, MCM2 and MCM5
30 proteins are much less abundant than are the MCM7 and MCM3 proteins. MCM6 forms a complex with MCM2, MCM4, and MCM7, which binds histone H3. In addition, the subcomplex of MCM4, MCM6, and MCM7 has helicase activity, which is mediated by the ATP-binding activity of MCM6 and the DNA-binding activity of MCM4. See, for example, Freeman *et al.* (1999) *Clin. Cancer Res.* 5:2121-2132; Lei

et al. (2001) *J. Cell Sci.* 114:1447-1454; Ishimi *et al.* (2003) *Eur. J. Biochem.* 270:1089-1101, all of which are herein incorporated by reference in their entirety.

Early publications have shown that the MCM proteins, and in particular, MCM-5, are useful for the detection of cervical disease (Williams *et al.* (1998) *Proc Natl Acad Sci U.S.A.* 95:14932-14937), as well as other cancers (Freeman *et al.* (1999) *Clin Cancer Res.* 5:2121-2132). The published literature indicates that antibodies to MCM-5 are capable of detecting cervical neoplastic cells. The specificity for detection of high-grade cervical disease has not been demonstrated for MCM-5 (Williams *et al.* (1998) *Proc Natl Acad Sci U.S.A.* 95:14932-14937). The detection of MCM-5 expression is not restricted to high-grade cervical disease but is also detected in identified low-grade dysplasia and proliferative cells that have re-entered the cell cycle following infection with high-risk HPV. In addition to MCM-5, other members from the MCM family, including MCM-2 and MCM-7 have been shown to be potentially useful markers for the detection of cervical neoplasia in tissue samples (Freeman *et al.* (1999) *Clin Cancer Res.* 5:2121-2132; Brake *et al.* (2003) *Cancer Res.* 63:8173-8180). Recent results have shown that MCM-7 appears to be a specific marker for the detection of high-grade cervical disease using immunochemistry formats (Brake *et al.* (2003) *Cancer Res.* 63:8173-8180; Malinowski *et al.* (2004) *Acta Cytol.* 43:696).

Therefore, there is a need in the art for antibodies that are capable of detecting expression of a biomarker that is selectively overexpressed in high-grade cervical disease. Such antibodies could be used in methods for differentiating high-grade disease from conditions that are not considered clinical disease, such as early-stage HPV infection and mild dysplasia.

25

SUMMARY OF THE INVENTION

Compositions and methods for diagnosing high-grade cervical disease are provided. Compositions include monoclonal antibodies capable of binding to nuclear biomarker proteins of the invention, particularly MCM proteins, more particularly MCM2. Antigen-binding fragments and variants of these monoclonal antibodies, hybridoma cell lines capable of producing these antibodies, and kits comprising the monoclonal antibodies of the invention are also encompassed herein.

The compositions of the invention find use in methods for diagnosing high-grade cervical disease. The methods comprise detecting overexpression of at least

one nuclear biomarker, wherein overexpression of the nuclear biomarker is indicative of high-grade cervical disease. Specifically, the methods comprise using the antibodies of the invention to detect overexpression of MCM2 in a cervical sample.

Compositions of the invention further include isolated polypeptides that
5 comprise an epitope capable of binding an MCM2 monoclonal antibody. These polypeptides find use in methods for producing MCM2 antibodies. Isolated nucleic acid molecules encoding the amino acid sequences of the MCM2 epitopes are also provided.

10 DETAILED DESCRIPTION OF THE INVENTION

Compositions and methods for diagnosing high-grade cervical disease are provided. Compositions include monoclonal antibodies that are capable of binding to nuclear biomarker proteins that are selectively overexpressed in high-grade cervical disease, particularly MCM proteins, more particularly MCM2. Hybridoma cell lines
15 that produce the monoclonal antibodies of the present invention are also disclosed. Kits comprising the monoclonal antibodies described herein are further provided. The present compositions find use in methods for diagnosing high-grade cervical disease in a patient.

The compositions of the invention include monoclonal antibodies that
20 specifically bind to MCM2, or to a variant or fragment thereof. In particular, the MCM2 antibodies designated as 27C5.6 and 26H6.19 are provided. Hybridoma cell lines that produce MCM2 monoclonal antibodies 27C5.6 and 26H6.19 were deposited with the Patent Depository of the American Type Culture Collection (ATCC), Manassas, Virginia, 20110-2209 on April 14, 2005 and assigned Patent Deposit Nos.
25 PTA-6668 and PTA-6667, respectively. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. § 112.

30 Antibodies that have the binding characteristics of monoclonal antibodies 27C5.6, and 26H6.19 are also disclosed herein. Such antibodies include, but are not limited to, antibodies that compete in competitive binding assays with these antibodies, as well as antibodies that bind to an epitope capable of binding monoclonal antibody 27C5.6 or 26H6.19. Variants and fragments of monoclonal

antibodies 27C5.6 and 26H6.19 that retain the ability to specifically bind to MCM2 are also provided. Compositions further include hybridoma cell lines that produce the monoclonal antibodies of the present invention and kits comprising at least one monoclonal antibody disclosed herein.

5 “Antibodies” and “immunoglobulins” (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to an antigen, immunoglobulins include both antibodies and other antibody-like molecules that lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

10 The terms “antibody” and “antibodies” broadly encompass naturally occurring forms of antibodies and recombinant antibodies such as single-chain antibodies, chimeric and humanized antibodies and multi-specific antibodies as well as fragments and derivatives of all of the foregoing, which fragments and derivatives have at least an antigenic binding site. Antibody derivatives may comprise a protein or chemical
15 moiety conjugated to the antibody. The term “antibody” is used in the broadest sense and covers fully assembled antibodies, antibody fragments that can bind antigen (e.g., Fab’, F’(ab)₂, Fv, single chain antibodies, diabodies), and recombinant peptides comprising the foregoing. As used herein, “MCM2 antibody” refers to any antibody that specifically binds to MCM2 (SEQ ID NO:1), or to a variant or fragment thereof,
20 and includes monoclonal antibodies, polyclonal antibodies, single-chain antibodies, and fragments thereof which retain the antigen binding function of the parent antibody.

 The MCM2 antibodies of the invention are optimally monoclonal antibodies. The term “monoclonal antibody” as used herein refers to an antibody obtained from a
25 population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts.

 “Native antibodies” and “native immunoglobulins” are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical
30 light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains.

Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to
5 form an interface between the light and heavy-chain variable domains.

The term “variable” refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It
10 is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR) regions. The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a p-sheet configuration, connected by three CDRs,
15 which form loops connecting, and in some cases forming part of, the p-sheet structure. The CDRs in each chain are held together in close proximity: by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site: of antibodies (see Kabat et al., NIH Publ. No.91-3242, Vol. I, pages 647-669 (1991)).

20 The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term “hypervariable region” when used herein refers to the amino acid residues of an antibody which: are responsible for antigen-binding. The hypervariable
25 region comprises amino acid residues from a “complementarily determining region” or “CDR” (i.e., residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institute of Health, i 25 Bethesda, MD. [1991])
30 and/or those residues from a “hypervariable loop” (i.e., residues 26-32(L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32(H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Clothia and Lesk, *J. Mol. Biol.*, 196:901-917 [1987]). “Framework” or “FR” residues are those variable domain residues other than the hypervariable region residues as herein deemed.

“Antibody fragments” comprise a portion of an intact antibody, preferably the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies (Zapata *et al.* (1995) *Protein Eng.* 8(10):1057-1062); single-chain antibody
5 molecules; and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called “Fab” fragments, each with a single antigen-binding site, and a residual “Fc” fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')₂ fragment that has two antigen-combining sites and is still capable of
10 cross-linking antigen.

“Fv” is the minimum antibody fragment that contains a complete antigen recognition and binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species, one heavy- and one light-chain variable
15 domain can be covalently linked by flexible peptide linker such that the light and heavy chains can associate in a “dimeric” structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody.
20 However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (C_{H1}) of the heavy chain. Fab fragments differ from Fab'
25 fragments by the addition of a few residues at the carboxy terminus of the heavy-chain C_{H1} domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them.

30 Fragments of the MCM2 antibodies are encompassed by the invention so long as they retain the desired affinity of the full-length antibody. Thus, for example, a fragment of an MCM2 antibody will retain the ability to bind to the MCM2 antigen. Such fragments are characterized by properties similar to the corresponding full-

length antibody, that is, the fragments will specifically bind MCM2. Such fragments are referred to herein as “antigen-binding” fragments.

Suitable antigen-binding fragments of an antibody comprise a portion of a full-length antibody, generally the antigen-binding or variable region thereof.

5 Examples of antibody fragments include, but are not limited to, Fab, F(ab')₂, and Fv fragments and single-chain antibody molecules. By “Fab” is intended a monovalent antigen-binding fragment of an immunoglobulin that is composed of the light chain and part of the heavy chain. By F(ab')₂ is intended a bivalent antigen-binding
10 fragment of an immunoglobulin that contains both light chains and part of both heavy chains. By “single-chain Fv” or “sFv” antibody fragments is intended fragments comprising the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. See, for example, U.S. Patent Nos. 4,946,778, 5,260,203, 5,455,030, and 5,856,456, herein incorporated by reference. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L
15 domains that enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun (1994) in *The Pharmacology of Monoclonal Antibodies*, Vol. 113, ed. Rosenberg and Moore (Springer-Verlag, New York), pp. 269-315.

Antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in, for example, McCafferty *et al.*
20 (1990) *Nature* 348:552-554 (1990) and U.S. Patent No. 5,514,548. Clackson *et al.* (1991) *Nature* 352:624-628 and Marks *et al.* (1991) *J. Mol. Biol.* 222:581-597 describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.* (1992) *Bio/Technology* 10:779-
25 783), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.* (1993) *Nucleic. Acids Res.* 21:2265-2266). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

Various techniques have been developed for the production of antibody
30 fragments. Traditionally, these fragments were derived *via* proteolytic digestion of intact antibodies (see, e.g., Morimoto *et al.* (1992) *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al.* (1985) *Science* 229:81). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries

discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter *et al.* (1992) *Bio/Technology* 10:163-167). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the
5 production of antibody fragments will be apparent to the skilled practitioner.

Preferably antibodies of the invention are monoclonal in nature. As indicated above, "monoclonal antibody" is intended an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be
10 present in minor amounts. The term is not limited regarding the species or source of the antibody. The term encompasses whole immunoglobulins as well as fragments such as Fab, F(ab')₂, Fv, and others which retain the antigen binding function of the antibody. Monoclonal antibodies are highly specific, being directed against a single antigenic site, i.e., a particular epitope within the MCM2 protein, as defined herein
15 below. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be
20 construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.* (1975) *Nature* 256:495, or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from
25 phage antibody libraries using the techniques described in, for example, Clackson *et al.* (1991) *Nature* 352:624-628; Marks *et al.* (1991) *J. Mol. Biol.* 222:581-597; and U.S. Patent No. 5,514,548.

Monoclonal antibodies can be prepared using the method of Kohler *et al.* (1975) *Nature* 256:495-496, or a modification thereof. Typically, a mouse is
30 immunized with a solution containing an antigen. Immunization can be performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally. Any method of immunization known in the art may be used to obtain the monoclonal antibodies of the invention. After immunization of the animal, the

spleen (and optionally, several large lymph nodes) are removed and dissociated into single cells. The spleen cells may be screened by applying a cell suspension to a plate or well coated with the antigen of interest. The B cells expressing membrane bound immunoglobulin specific for the antigen (i.e., antibody-producing cells) bind to the plate and are not rinsed away. Resulting B cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form monoclonal antibody-producing hybridomas, and are cultured in a selective medium. The resulting cells are plated by serial dilution and are assayed for the production of antibodies that specifically bind the antigen of interest (and that do not bind to unrelated antigens). The selected monoclonal antibody (mAb)-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice). Monoclonal antibodies can also be produced using Repetitive Immunizations Multiple Sites technology (RIMMS). See, for example, Kilpatrick *et al.* (1997) *Hybridoma* 16(4):381-389; Wring *et al.* (1999) *J. Pharm. Biomed. Anal.* 19(5):695-707; and Bynum *et al.* (1999) *Hybridoma* 18(5):407-411, all of which are herein incorporated by reference in their entirety.

As an alternative to the use of hybridomas, antibody can be produced in a cell line such as a CHO cell line, as disclosed in U.S. Patent Nos. 5,545,403; 5,545,405; and 5,998,144; incorporated herein by reference. Briefly the cell line is transfected with vectors capable of expressing a light chain and a heavy chain, respectively. By transfecting the two proteins on separate vectors, chimeric antibodies can be produced. Another advantage is the correct glycosylation of the antibody. A monoclonal antibody can also be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with a biomarker protein to thereby isolate immunoglobulin library members that bind the biomarker protein. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP 9 Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication Nos. WO 92/18619; WO 91/17271; WO 92/20791; WO 92/15679; 93/01288; WO 92/01047; 92/09690; and 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum.*

Antibod. Hybridomas 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J.* 12:725-734.

In some aspects of the invention, antibodies may be selected on the basis of desirable staining of cytological, rather than histological, samples. That is, in particular embodiments the antibodies are selected with the end sample type (e.g., cytology preparations) in mind and for binding specificity. Antibodies directed to specific biomarkers of interest, such as MCM2, are selected and purified via a multi-step screening process. Such methods for antibody selection are described in pending U.S. Application Serial No. 11/087,227, entitled "Methods and Compositions for the Detection of Cervical Disease," filed March 23, 2005, which is herein incorporated by reference in its entirety.

Antibodies having the binding characteristics of a monoclonal antibody of the invention are also provided. "Binding characteristics" or "binding specificity" when used in reference to an antibody means that the antibody recognizes the same or similar antigenic epitope as a comparison antibody. Examples of such antibodies include, for example, an antibody that competes with a monoclonal antibody of the invention in a competitive binding assay. One of skill in the art could determine whether an antibody competitively interferes with another antibody using standard methods.

By "epitope" is intended the part of an antigenic molecule to which an antibody is produced and to which the antibody will bind. An "MCM2 epitope" comprises the part of the MCM2 protein to which an MCM2 monoclonal antibody binds. Epitopes can comprise linear amino acid residues (i.e., residues within the epitope are arranged sequentially one after another in a linear fashion), nonlinear amino acid residues (referred to herein as "nonlinear epitopes"; these epitopes are not arranged sequentially), or both linear and nonlinear amino acid residues. Typically epitopes are short amino acid sequences, e.g. about five amino acids in length. Systematic techniques for identifying epitopes are known in the art and are described, for example, in U.S. Pat. No. 4,708,871 and in the examples set forth below. Briefly, in one method, a set of overlapping oligopeptides derived from the antigen may be synthesized and bound to a solid phase array of pins, with a unique oligopeptide on each pin. The array of pins may comprise a 96-well microtiter plate, permitting one to assay all 96 oligopeptides simultaneously, e.g., for binding to a biomarker-specific monoclonal antibody. Alternatively, phage display peptide library kits (New England

BioLabs) are currently commercially available for epitope mapping. Using these methods, the binding affinity for every possible subset of consecutive amino acids may be determined in order to identify the epitope that a given antibody binds. Epitopes may also be identified by inference when epitope length peptide sequences
5 are used to immunize animals from which antibodies are obtained.

The invention also encompasses isolated polypeptides comprising an epitope for binding an MCM2 monoclonal antibody. These polypeptides correspond to a portion of the antigen (i.e., MCM2) that binds to a monoclonal antibody. Such polypeptides find use in methods for producing antibodies that bind selectively to
10 MCM2. The ability of a polypeptide to be used in the production of antibodies is referred to herein as "antigenic activity." For example, the amino acid sequences set forth in SEQ ID NOs: 3, 4, and 14 (corresponding to residues 369 to 382, 688 to 710, and 683 to 692, respectively, in the MCM2 amino acid sequence set forth in SEQ ID NO:1) comprise epitopes recognized by MCM2 monoclonal antibodies, more
15 particularly monoclonal antibodies 27C5.6 and 26H6.19. See Example 4 for details. Variants and fragments of the MCM2 epitope sequences set forth in SEQ ID NOs: 3, 4, and 14 that retain the antigenic activity of the original polypeptide are also provided. The invention further includes isolated nucleic acid molecules that encode polypeptides that comprise MCM2 epitopes, and variants and fragments thereof.

20 The polypeptides of the invention comprising MCM2 epitopes can be used in methods for producing monoclonal antibodies that specifically bind to MCM2, as described herein above. Such polypeptides can also be used in the production of polyclonal MCM2 antibodies. For example, polyclonal antibodies can be prepared by immunizing a suitable subject (e.g., rabbit, goat, mouse, or other mammal) with a
25 polypeptide comprising an MCM2 epitope (i.e., an immunogen). The antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized biomarker protein. At an appropriate time after immunization, e.g., when the antibody titers are highest, antibody-producing cells can be obtained from the subject and used to
30 prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497, the human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985) in *Monoclonal Antibodies and*

Cancer Therapy, ed. Reisfeld and Sell (Alan R. Liss, Inc., New York, NY), pp. 77-96) or trioma techniques. The technology for producing hybridomas is well known (see generally Coligan *et al.*, eds. (1994) *Current Protocols in Immunology* (John Wiley & Sons, Inc., New York, NY); Galfre *et al.* (1977) *Nature* 266:55052; Kenneth (1980) in
5 *Monoclonal Antibodies: A New Dimension In Biological Analyses* (Plenum Publishing Corp., NY; and Lerner (1981) *Yale J. Biol. Med.*, 54:387-402).

Amino acid sequence variants of a monoclonal antibody or a polypeptide comprising an MCM2 epitope described herein are also encompassed by the present invention. Variants can be prepared by mutations in the cloned DNA sequence
10 encoding the antibody of interest. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol.* 154:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A*
15 *Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the polypeptide of interest may be found in the model of Dayhoff *et al.* (1978) in *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.),
20 herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred. Examples of conservative substitutions include, but are not limited to, Gly \leftrightarrow Ala, Val \leftrightarrow Ile \leftrightarrow Leu, Asp \leftrightarrow Glu, Lys \leftrightarrow Arg, Asn \leftrightarrow Gln, and Phe \leftrightarrow Trp \leftrightarrow Tyr.

In constructing variants of the polypeptide of interest, modifications are made
25 such that variants continue to possess the desired activity, i.e., similar binding affinity to the biomarker. Obviously, any mutations made in the DNA encoding the variant polypeptide must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

30 Preferably, variants of a reference polypeptide have amino acid sequences that have at least 70% or 75% sequence identity, preferably at least 80% or 85% sequence identity, more preferably at least 90%, 91%, 92%, 93%, 94% or 95% sequence identity to the amino acid sequence for the reference antibody molecule, or to a

shorter portion of the reference antibody molecule. More preferably, the molecules share at least 96%, 97%, 98% or 99% sequence identity. For purposes of the present invention, percent sequence identity is determined using the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12
5 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is taught in Smith and Waterman (1981) *Adv. Appl. Math.* 2:482-489. A variant may, for example, differ from the reference antibody by as few as 1 to 15 amino acid residues, as few as 1 to 10 amino acid residues, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

10 With respect to optimal alignment of two amino acid sequences, the contiguous segment of the variant amino acid sequence may have additional amino acid residues or deleted amino acid residues with respect to the reference amino acid sequence. The contiguous segment used for comparison to the reference amino acid sequence will include at least 20 contiguous amino acid residues, and may be 30, 40,
15 50, or more amino acid residues. Corrections for sequence identity associated with conservative residue substitutions or gaps can be made (see Smith-Waterman homology search algorithm).

The MCM2 monoclonal antibodies of the invention may be labeled with a detectable substance as described below to facilitate biomarker protein detection in
20 the sample. Such antibodies find use in practicing the methods of the invention. The antibodies and antibody fragments of the invention can be coupled to a detectable substance to facilitate detection of antibody binding. The word "label" when used herein refers to a detectable compound or composition that is conjugated directly or indirectly to the antibody so as to generate a "labeled" antibody. The label may be
25 detectable by itself (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition that is detectable. Examples of detectable substances for purposes of labeling antibodies include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples
30 of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate,

rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , or ^3H .

5 Kits comprising at least one MCM2 monoclonal antibody of the invention are further provided. By "kit" is intended any manufacture (e.g., a package or a container) comprising at least one reagent, i.e., an antibody, for specifically detecting the expression of MCM2. The kit may be promoted, distributed, or sold as a unit for performing the methods of the present invention. Additionally, the kits may contain a
10 package insert describing the kit and methods for its use.

 Kits of the invention generally comprise at least one monoclonal antibody directed to MCM2, chemicals for the detection of antibody binding, a counterstain, and, optionally, a bluing agent to facilitate identification of positive staining cells. Any chemicals that detect antigen-antibody binding may be used in the kits of the
15 invention. In some embodiments, the detection chemicals comprise a labeled polymer conjugated to a secondary antibody. For example, a secondary antibody that is conjugated to an enzyme that catalyzes the deposition of a chromogen at the antigen-antibody binding site may be provided. Such enzymes and techniques for using them in the detection of antibody binding are well known in the art. In one embodiment,
20 the kit comprises a secondary antibody that is conjugated to an HRP-labeled polymer. Chromogens compatible with the conjugated enzyme (e.g., DAB in the case of an HRP-labeled secondary antibody) and solutions, such as hydrogen peroxide, for blocking non-specific staining may be further provided. In other embodiments, antibody binding to a biomarker protein is detected through the use of a mouse probe
25 reagent that binds to monoclonal antibodies, followed by addition of a dextran polymer conjugated with HRP that binds to the mouse probe reagent. Such detection reagents are commercially available from, for example, Biocare Medical.

 The kits of the present invention may further comprise a peroxidase blocking reagent (e.g., hydrogen peroxide), a protein blocking reagent (e.g., purified casein),
30 and a counterstain (e.g., hematoxylin). A bluing agent (e.g., ammonium hydroxide or TBS, pH 7.4, with Tween-20 and sodium azide) may be further provided in the kit to facilitate detection of positive staining cells. Kits may also comprise positive and negative control samples for quality control purposes.

In another embodiment, the kits of the invention comprise two MCM2 monoclonal antibodies, more particularly monoclonal antibodies 27C5.6 and 26H6.19. A kit comprising two MCM2 monoclonal antibodies and a third antibody directed to topoisomerase II alpha (Topo2A) is further provided. When multiple
5 antibodies are present in the kit, each antibody may be provided as an individual reagent or, alternatively, as an antibody cocktail comprising all of the antibodies of interest. Furthermore, any or all of the kit reagents may be provided within containers that protect them from the external environment, such as in sealed containers. The kits of the invention are useful in the diagnosis of high-grade cervical disease and may
10 further include reagents for Pap staining (e.g., EA50 and Orange G).

The compositions of the invention find use in methods for diagnosing high-grade cervical disease in a patient such as those disclosed in pending U.S. Application Serial No. 11/087,227, entitled "Methods and Compositions for the Detection of Cervical Disease," filed March 23, 2005, which is herein incorporated by reference in
15 its entirety. "Diagnosing high-grade cervical disease" is intended to include, for example, diagnosing or detecting the presence of cervical disease, monitoring the progression of the disease, and identifying or detecting cells or samples that are indicative of high-grade cervical disease. The terms diagnosing, detecting, and identifying high-grade cervical disease are used interchangeably herein. By "high-
20 grade cervical disease" is intended those conditions classified by colposcopy as premalignant pathology, malignant pathology, moderate to severe dysplasia, and cervical cancer. Underlying high-grade cervical disease includes histological identification of CINII, CINIII, HSIL, carcinoma *in situ*, adenocarcinoma, and cancer (FIGO stages I-IV).

The methods of the invention comprise detecting overexpression of at least
25 one nuclear biomarker that is selectively overexpressed in high-grade cervical disease. By "nuclear biomarker" is intended any gene or protein that is predominantly expressed in the nucleus of the cell. A nuclear biomarker may be expressed to a lesser degree in other parts of the cell. By "selectively overexpressed in high-grade
30 cervical disease" is intended that the nuclear biomarker of interest is overexpressed in high-grade cervical disease but is not overexpressed in conditions classified as LSIL, CINI, HPV-infected samples without any dysplasia present, immature metaplastic cells, and other conditions that are not considered to be clinical disease. Thus, detection of the nuclear biomarkers of the invention permits the differentiation of

samples indicative of underlying high-grade cervical disease from samples that are indicative of benign proliferation, early-stage HPV infection, or mild dysplasia. Nuclear biomarkers of particular interest include MCM proteins, particularly MCM2, and Topo2A.

5 In a particular aspect of the invention, the methods comprise obtaining a cervical sample from a patient, contacting the sample with at least one MCM2 monoclonal antibody of the invention, and detecting binding of the antibody to MCM2. In other embodiments, the sample is contacted with at least two monoclonal antibodies that specifically bind to MCM2, particularly monoclonal antibodies 27C5.6
10 and 26H6.19. In a further embodiment, the sample is contacted with these two MCM2 monoclonal antibodies and a third antibody that specifically binds to Topo2A. Techniques for detecting antibody binding are well known in the art. Antibody binding to a biomarker of interest may be detected through the use of chemical reagents that generate a detectable signal that corresponds to the level of antibody
15 binding and, accordingly, to the level of biomarker protein expression. Any method for detecting antibody-antigen binding may be used to practice the methods of the invention.

 As used herein, "cervical sample" refers to any sampling of cells, tissues, or bodily fluids from the cervix in which expression of a biomarker can be detected.
20 Examples of such body samples include but are not limited to gynecological fluids, biopsies, and smears. Cervical samples may be obtained from a patient by a variety of techniques including, for example, by scraping or swabbing an area or by using a needle to aspirate bodily fluids. Methods for collecting cervical samples are well known in the art. In particular embodiments, the cervical sample comprises cervical
25 cells, particularly in a liquid-based preparation. In one embodiment, cervical samples are collected according to liquid-based cytology specimen preparation guidelines such as, for example, the SurePath® (TriPath Imaging, Inc.) or the ThinPrep® preparation (CYTYC, Inc.). Cervical samples may be transferred to a glass slide for viewing under magnification. Fixative and staining solutions may be applied to the cells on
30 the glass slide for preserving the specimen and for facilitating examination. In one embodiment the cervical sample will be collected and processed to provide a monolayer sample, as set forth in US Patent No. 5,346,831, herein incorporated by reference.

One of skill in the art will appreciate that any or all of the steps in the methods of the invention could be implemented by personnel in a manual or automated fashion. Thus, the steps of cervical sample preparation, antibody, and detection of antibody binding may be automated. The methods of the invention may also be
5 combined with conventional Pap staining techniques to permit a more accurate diagnosis of high-grade cervical disease.

The following examples are offered by way of illustration and not by way of limitation:

10

EXPERIMENTAL

Example 1: Production of Mouse Monoclonal Antibodies to MCM2

Mouse monoclonal antibodies specific for MCM2 were generated. The antigen (an immunogenic polypeptide) was a full-length recombinant hexahistidine-tagged MCM2 protein. The antigen was expressed using a baculovirus expression
15 system in Tni cells. Specifically, the coding sequence for the hexahistidine-tagged MCM2 (SEQ ID NO:10) was cloned into the pFastBac1 plasmid (Invitrogen) for expression in Tni cells. Methods for producing recombinant proteins using baculovirus expression systems are well known in the art. The tagged MCM2 protein
20 was purified using a chelating agarose charged with Ni⁺² ions (Ni-NTA from Qiagen) and used as an immunogen. The amino acid sequence of the immunogenic MCM2 polypeptide is provided in SEQ ID NO:11.

Mouse immunizations and hybridoma fusions were performed essentially as described in Kohler *et al.* (1975) *Nature* 256:495-496. Mice were immunized with
25 the immunogenic tagged-MCM2 protein in solution. Antibody-producing cells were isolated from the immunized mice and fused with myeloma cells to form monoclonal antibody-producing hybridomas. The hybridomas were cultured in a selective medium. The resulting cells were plated by serial dilution and assayed for the production of antibodies that specifically bind MCM2 (and that do not bind to
30 unrelated antigens). To confirm that the monoclonal antibodies of interest reacted with the MCM2 protein only and not with the hexahistidine tag, selected hybridomas were screened against an MCM2-FLAG-tagged protein. The nucleotide and amino acid sequences for the MCM2-FLAG protein are set forth in SEQ ID NOs:12 and 13,

respectively. Selected monoclonal antibody (mAb)-secreting hybridomas were then cultured.

Antibodies were purified from the culture media supernatants of “exhausted” hybridoma cells (i.e., cells grown until viability drops to between 0-15%) using
5 recombinant Protein A-coated resin (STREAMLINE®, Amersham, Inc.). Antibodies were eluted using low pH followed by immediate neutralization of pH. Fractions with significant absorbances at 280 nM were pooled. The resultant pool was dialyzed against PBS. Purified antibodies were subjected to further characterization. MCM2 monoclonal antibodies 26H6.19 and 27C5.6 were both determined to be IgG₁
10 isotypes. Details of the epitope mapping of these antibodies are described below.

Example 2: Isolation of Monoclonal Antibodies from Hybridoma Cells

The following procedure is used to isolate monoclonal antibodies from hybridoma cells:

15 *Media preparation*

- To a sterile 1,000 ml storage bottle, add 100 ml Hyclone Fetal Bovine Serum (FBS).
- Add 10 ml of MEM Non-Essential Amino Acids Solution.
- Add 10 ml of Penicillin-Streptomycin-L-Glutamine Solution.
- 20 • QS to approximately 1000 ml with ExCell 610-HSF media.
- Place sterile cap on bottle and secure tightly. Swirl gently to mix.
- Connect a 1000 ml sterile acetate vacuum filter unit (0.2 µm) to a vacuum pump system.
- Gently pour approximately half of the media solution into sterile acetate
25 vacuum filter unit and turn on the vacuum.
- Once the first half of the media has been filtered, pour the remaining media into the filter unit and continue filtering.
- After all the media has been filtered, disconnect the vacuum hose from the vacuum filter unit and turn off the vacuum pump. Remove the receiver
30 portion of the filter unit from the filter bottle. Place a new sterile bottle cap on the bottle.
- Store at 2°C to 10°C. Protect from light.

Initial hybridoma cell culture

- Thaw vial of stock hybridoma frozen culture in a pre-warmed 37°C H₂O bath.
- Spray the outside of the freeze vial with 70% ethanol.
- Move the thawed vial into the Biological Safety Cabinet.
- 5 • Remove the cells from the freeze vial and transfer the cells to a 15 ml centrifuge tube.
- Add 7 ml of cell culture media drop-wise to the 15 ml centrifuge tube containing the thawed cells.
- Centrifuge the 15 ml centrifuge tube containing the thawed cells and culture media for 5 minutes at 200 g force.
- 10 • While the cells are in the centrifuge, add 45 ml of cell culture media to a sterile T-225 flask.
- After centrifugation, visually inspect the tube for the presence of a cell pellet.
- Remove the media from the centrifuge tube being careful not to dislodge the cell pellet. Note: If the cell pellet is disturbed, repeat the centrifugation step.
- 15 • Add 5 ml of cell culture media to the 15 ml centrifuge tube containing the pelleted cells. Pipette to re-suspend the cell pellet into the media.
- Transfer the entire contents of the resuspended cells and culture media into the T-225 flask containing the 45 ml of media.
- 20 • Cap the T-225 flask.
- Observe for presence of intact cells under the microscope. Place the T-225 flask immediately into a CO₂ incubator and allow the cells to incubate overnight.

25 *Expansion of hybridoma cell line*

- Continue to monitor the cell culture for viability, concentration, and presence of contamination.
- Monitor and adjust the cell suspension from the initial T-225 flask until the concentration is approximately 600,000 cells/ml to 800,000 cells/ml and a total of 200 to 250 ml of media.
- 30 • Dislodge cells and add additional media as needed to meet minimum cell density requirements. Divide and transfer cell suspension into one new sterile T-225 flask. Place the 2 x T-225 flasks into the CO₂ incubator.

- Monitor the cells from the 2 x T-225 flasks until the concentration is approximately 600,000 cells/ml to 800,000 cells/ml, and a total of between 200 to 250 ml of media for each flask.
- 5 • Dislodge cells and add additional media as needed to meet minimum cell density requirements. Divide and transfer the cell suspensions into 2 additional new sterile T-225 flasks for a total of 4 x T-225 flasks. Return all flasks to the CO2 incubator.
- 10 • Monitor the cells, and adjust volume in the 4 x T-225 flasks until the cell concentration is approximately 600,000 cells/ml to 800,000 cells / ml with a total volume of approximately 250 ml per T-225 flask (or approximately 1000 ml total).
- 15 • Continue to monitor the cells from the 4 x T-225 flasks until the cells have grown to exhaustion, with a final viability of 0%-15%. The cell culture supernatant is now ready for the Clarification Process.

15

Clarification of supernatant

- Turn on the tabletop centrifuge. Place the 500 ml tube adapters into the rotor buckets, close the lid and set the temperature to 4°C (+/-) 4°C.
- 20 • Using aseptic technique, pour the media from all four of the now exhausted T-225 flasks into 2 x 500 ml conical centrifuge tubes.
- Make sure the 2 x 500 ml tubes are balanced. Transfer supernatant from one tube to the other as necessary to balance them.
- Centrifuge the exhausted supernatant at 1350 g (+/- 40 g) for 15 minutes at 2°C to 10°C.
- 25 • After centrifugation is complete, aseptically decant the supernatant into a sterile 1000 ml storage bottle and secure with a sterile cap.
- Aseptically transfer 1 ml to the microfuge tube. Store microfuge tube with sample at 2°C to 10°C (Protect from light).
- 30 • The clarified supernatant sample is ready for IgG evaluation using the Easy-Titer® Assay.

*Buffer preparation*Binding buffer:

- Add approximately 600 ml of DI H₂O to a clean beaker.
- 5 • Add 77.28 ml of Boric Acid solution (4% W/V). Stir at room temperature with a clean stir bar.
- Weigh out 233.76 g of Sodium Chloride and place into the solution while continuing to stir.
- Bring solution up to approximately 950 ml with DI H₂O and continue to stir.
- 10 • When the Sodium Chloride has dissolved and the solution is clear, adjust the pH to 9.0 ± 0.2 with Sodium Hydroxide.
- Remove the solution to a clean 1000 ml graduated cylinder and QS to 1000 ml with DI H₂O.
- Transfer the completed buffer to an appropriate storage bottle. This buffer may be stored for up to 7 days before use.
- 15 • Repeat this entire process to prepare an additional 0.2 liters to 1.0 liter of Binding Buffer.

Elution buffer

- 20 • Weigh out 1.725 g of sodium phosphate, monobasic and place into a clean 250 ml beaker with a clean stir bar.
- Weigh out 3.676 g of sodium citrate and place into the same clean 250 ml beaker.
- Add approximately 175 ml of DI H₂O and stir at room temperature until dissolved.
- 25 • Weigh out 4.38 g of Sodium Chloride and place into the solution while continuing to stir.
- Bring solution up to approximately 225 ml with DI H₂O and continue to stir.
- When the Sodium Chloride has dissolved and the solution is clear, adjust the pH to 3.5 ± 0.2 with Hydrochloric Acid.
- 30 • Remove the solution to a clean 250 ml graduated cylinder and QS to 250 ml with DI H₂O.
- Connect a 500 ml sterile acetate vacuum filter unit (0.2 μ m) to a vacuum pump system and filter sterilize the solution.

- Remove the filter and close the container with a sterile cap.

Antibody Adsorption

- 5 • Pour the Clarified Supernatant (~1L) into a clean 4000 ml plastic beaker with a clean stir bar.
- Add an approximately equal amount (~1L) of the Binding Buffer to the clean 4000 ml plastic beaker containing the clarified supernatant. Add a clean stir bar.
- Cover the beaker with clean plastic wrap and label "Antibody Binding."
- 10 • Calculate the approximate amount of STREAMLINE® Protein A that will be needed using the data in Table 1.

Table 1: Volume of Protein A Resin Required

Quantity IgG (μg /ml) in Supernatant	Volume of Protein A Resin Required in Milliliters (ml)
>180 - \leq 200	12.0
>160 - \leq 180	11.0
>140 - \leq 160	10.0
>120 - \leq 140	9.0
>100 - \leq 120	8.0
>80 - \leq 100	7.0
>60 - \leq 80	6.0
>40 - \leq 60	4.5
>20 - \leq 40	3.5
\leq 20	2.0

- 15 • Secure a clean Disposable Column and stopcock assembly to a ring stand and clamp. Close the stopcock.
- Mix appropriate amount of STREAMLINE Protein A beads by inverting the bottle several times. Withdraw the required volume and place into the Disposable Column.
- 20 • Wash the STREAMLINE Protein A beads with 10 ml of DI H₂O. Open the stopcock and allow the DI H₂O to drain. Close the stopcock. Repeat with an additional 10 ml of DI H₂O.
- Wash the STREAMLINE Protein A beads with 10 ml of Binding Buffer. Open the stopcock and allow the Binding Buffer to drain. Close the stopcock. Repeat with an additional 10 ml of Binding Buffer.

- Resuspend the STREAMLINE Protein A beads in ~10 ml of the Clarified Supernatant and Binding Buffer solution (from the 4000 ml beaker) and transfer the beads into the 4000 ml beaker containing the Clarified Supernatant and Binding Buffer solution. Repeat as required to transfer any remaining
5 beads. When completed, discard the column and stopcock.
- Allow the mixture to mix vigorously at 2°C to 10°C for approximately 18 hours.
- When mixing is complete, turn off the stir plate and remove the “Antibody Binding” beaker with the buffered supernatant and bead suspension back to
10 the lab bench area. Allow the STREAMLINE Protein A beads to settle to the bottom of the beaker (approximately 5 minutes).
- Secure a clean Disposable Column and stopcock assembly to a ring stand and clamp. Close the stopcock.
- Label a clean, 250 ml bottle or suitable container “Column Wash-Post
15 Binding.”
- Label a clean plastic beaker “Supernatant-Post Binding.”
- Decant the supernatant from the 4000 ml beaker into the clean, labeled, 2 liter plastic beaker, leaving the beads in the bottom of the 4000 ml beaker. Cover the 2000 ml beaker containing the “Supernatant-Post Binding” solution with
20 clean plastic wrap and store at 2°C to 10°C.
- Add approximately 15 ml of Binding Buffer into the decanted 4000 ml “Antibody Binding” beaker. Resuspend the STREAMLINE Protein A beads and transfer them to the column. Open the stopcock and allow the Binding Buffer to drain into the “Column Wash-Post binding” container. Close the
25 stopcock when drained.
- Transfer any remaining STREAMLINE Protein A beads in the “Antibody Binding” beaker by adding additional Binding Buffer, mixing, and transferring to the column as in the preceding steps. Close the stopcock when drained.
- Calculate the approximate amount of Binding Buffer needed to wash the
30 STREAMLINE Protein A beads in the column using the data in Table 2.

Table 2: Binding Buffer Volume for Column Wash

Quantity IgG (μg /ml) in Supernatant	Volume of Binding Buffer Required in Milliliters (ml)
> 180 - \leq 200	5 column washes total with 15.0 ml each
> 160 - \leq 180	5 column washes total with 15.0 ml each
> 140 - \leq 160	5 column washes total with 12.5 ml each
> 120 - \leq 140	5 column washes total with 12.5 ml each
> 100 - \leq 120	5 column washes total with 12.5 ml each
> 80 - \leq 100	5 column washes total with 10.0 ml each
> 60 - \leq 80	5 column washes total with 10.0 ml each
> 40 - \leq 60	5 column washes total with 7.5 ml each
> 20 - \leq 40	5 column washes total with 5.0 ml each
\leq 20	5 column washes total with 5.0 ml each

- Wash the STREAMLINE Protein A beads in the column with the appropriate volume of Binding Buffer for the appropriate number of washes, continuing to collect the effluent into the “Column Wash-Post Binding” container.
- 5
- When completed, close the stopcock. Store the “Column Wash-Post Binding” container at 2°C to 10°C.
 - Determine the Total Volumes of Elution Buffer and Neutralization Buffer needed to elute the STREAMLINE Protein A beads in the column from Table 3.

Quantity IgG ($\mu\text{g/ml}$) in Supernatant	Total Volume of Elution Buffer Required (ml)	Total Volume of Neutralization Buffer Required (ml)	Volume of Elution Buffer Required <u>per fraction</u> (ml)	Volume of Neutralization Buffer Required <u>per fraction</u> (ml)
> 180 - \leq 200	72	7.2	12	1.2
> 160 - \leq 180	66	6.6	11	1.1
> 140 - \leq 160	60	6.0	10	1.0
> 120 - \leq 140	54	5.4	9	0.9
> 100 - \leq 120	48	4.8	8	0.8
> 80 - \leq 100	42	4.2	7	0.7
> 60 - \leq 80	36	3.6	6	0.6
> 40 - \leq 60	27	2.7	4.5	0.45
> 20 - \leq 40	21	2.1	3.5	0.35
\leq 20	12	1.2	2	0.2

- Label 9 sterile conical centrifuge tubes “Eluted Antibody”, Fraction # (1 through 9).
- Place the appropriate volume of Neutralization Buffer required per fraction (as determined from Table “C” above) into each of the 9 “Eluted Antibody” fraction tubes and place securely under the column stopcock outlet.
- Elute the STREAMLINE Protein A beads in the column fraction by fraction with the appropriate volume of Elution Buffer required per fraction (as determined from Table 3 above) while collecting the eluate into each of the “Eluted Antibody” tubes containing Neutralization Buffer.
- When the elutions are complete, mix each “Eluted Antibody” fraction tube gently by swirling several times. Remove approximately 50 μl of fraction # 3 and place on a pH test paper strip to ensure that the eluate has been neutralized to an approximate pH between 6.5 to 8.5. If required, add additional Neutralizing Buffer or Elution Buffer as needed to bring pH into range.
- When pH evaluation is completed, perform an Absorbance Scan of a sample from each fraction at 280 nm – 400 nm to determine the approximate concentration of IgG in the eluate prior to proceeding to the Dialysis Process.

Accept fractions as part of the Eluate Pool if the A280-A400 value is \geq 0.200.

Reject fractions as part of the Eluate Pool if the A280-A400 value is $<$ 0.200.

- 5
 - Label a sterile conical centrifuge tube “Eluted Antibody,” “Eluate Pool,” and combine all fractions that were Accepted as part of the pool.
 - Perform an Absorbance Scan of a sample of the Eluate Pool to determine the approximate concentration of IgG in the eluate prior to proceeding to the Dialysis Process.
- 10
 - Estimate the volume of the Eluate Pool and calculate the approximate total mgs of IgG.
 - Volume of Eluate Pool: _____ mls x _____ IgG mg/ml = _____ Total mgs of IgG
- 15 *Antibody dialysis*
 - Remove the “Eluted Antibody” tube from 2°C to 10°C.
 - Calculate the approximate length of Dialysis Tubing that will be needed to dialyze the antibody eluate using the approximate volume of eluate and the data in Table 4.

Approximate Volume of Eluent (ml)	Volume/length Ratio of Dialysis Tubing	Approximate Length Needed for Eluent Sample (cm)	Head Space of 20% (cm)	Approximate Length Needed for Sample plus Headspace (cm)	Approximate Length Needed for Tie Off of Tubing (cm)	Approximate Total Length of Dialysis Tubing Needed (cm)
39.6	2	20	4	24	15	63
36.3	2	18	4	22	15	59
33.0	2	17	3	20	15	55
29.7	2	15	3	18	15	51
26.4	2	13	3	16	15	47
23.1	2	12	2	14	15	43
19.8	2	10	2	12	15	39
14.85	2	7	1	9	15	33
11.55	2	6	1	7	15	29
6.6	2	3	1	4	15	23

- 5
- Cut the appropriate length of dialysis tubing required. (Spectra/Por® 2 Regenerated Cellulose Membrane, 12,000 – 14,000 Dalton Molecular Weight Cutoff (MWCO), 16 mm Diameter, Spectrum Laboratories Inc., Cat. No. 132678)
 - Hydrate the dialysis membrane tubing in 1000 ml of DIH₂O for > 30 minutes.
 - Calculate the approximate volume of Dialysis Buffer needed to dialyze the antibody eluate using the data in Table 5.

Table 5: Volume of Dialysis Buffer Required

Quantity IgG (µg/ml) in Supernatant	Final Volume of Eluted Antibody in Milliliters (ml)	Length of Dialysis Tubing Needed (cm)	Volume of Dialysis Buffer (1 X PBS) Needed in Liters
> 180 - ≤200	39.6 ml	63 cm	3 complete changes of 4.0 Liters
> 160 - ≤180	36.3 ml	59 cm	3 complete changes of 3.6 Liters
> 140 - ≤160	33.0 ml	55 cm	3 complete changes of 3.3 Liters
> 120 - ≤140	29.7 ml	51 cm	3 complete changes of 3.0 Liters
> 100 - ≤120	26.4 ml	47 cm	3 complete changes of 2.6 Liters
> 80 - ≤100	23.1 ml	43 cm	3 complete changes of 2.3 Liters
> 60 - ≤80	19.8 ml	39 cm	3 complete changes of 1.9 Liters
> 40 - ≤60	14.85 ml	33 cm	3 complete changes of 1.5 Liters
> 20 - ≤40	11.55 ml	29 cm	3 complete changes of 1.2 Liters
≤20	6.6 ml	23 cm	3 complete changes of 0.7 Liters

10

- Place the appropriate amount of Dialysis Buffer into a suitable sized plastic beaker. Label the beaker “Dialyzed Antibody.” Add a clean stir bar and place the beaker on a stir plate inside a refrigerator or cold room at 2°C to 10°C.
 - Rinse the dialysis tubing thoroughly in DI-H₂O. Tie two end knots approximately 7 cm from one end of the dialysis tubing and secure tightly.
 - Add approximately 5 ml of DI-H₂O into the dialysis tubing.
 - Fill the dialysis tubing with the eluted antibody from the “Eluted Antibody” collection tube.
- 15

- Tie two end knots approximately 7 cm from the remaining open end of the dialysis tubing and secure tightly. Ensure that the headspace is approximately that as derived from Table 4.
- 5 • Place the filled and closed dialysis tubing into the dialysis reservoir with the appropriate volume of 1X PBS (from Table 5).
- Cover the beaker with clean plastic wrap. Adjust the speed on the stir plate such that the dialysis sample spins freely, but is not pulled down into the vortex of the dialysate. Dialysis should take place at 2°C to 10°C with 3 buffer exchanges in total within a 24 hour period.

10

Antibody filtration

- Label a sterile collection tube “Dialyzed Antibody.”
- Remove the dialyzed sample tubing from the dialysis beaker. Cut the dialysis tubing open at one end and transfer the dialyzed sample into the “Dialyzed
15 Antibody” centrifuge tube.
- Label another sterile collection tube “Dialyzed Antibody.”
- Select a sterile Luer Lok syringe with adequate capacity to hold the final dialyzed volume.
- Attach an Acrodisc® Syringe Filter to the opening of the syringe (0.2 µm HT
20 Tuffryn® Membrane, Low Protein binding, Gelman Laboratories, Cat. No. 4192). Remove the plunger from the syringe and while holding the syringe upright, transfer the dialyzed monoclonal antibody from the “Dialyzed Antibody” tube into the syringe. Replace the plunger.
- Hold the Acrodisc® Syringe Filter over the opened, sterile, labeled “Purified
25 Antibody” collection tube, and depress the syringe plunger to filter the purified antibody into the “Purified Antibody” tube.
- When filtration is complete, cap the “Purified Antibody” tube and store at 2°C to 10°C.
- Determine concentration of purified monoclonal antibody using A280
30 procedure.

Example 3: General Method for Epitope Mapping

General Approach

Epitope mapping is performed to identify the linear amino acid sequence within an antigenic protein (i.e., the epitope) that is recognized by a particular
5 monoclonal antibody. A general approach for epitope mapping requires the expression of the full-length protein, as well as various fragments (i.e., truncated forms) of the protein, generally in a heterologous expression system. These various recombinant proteins are then used to determine if the specific monoclonal antibody is capable of binding one or more of the truncated forms of the target protein. Through
10 the use of reiterative truncation and the generation of recombinant proteins with overlapping amino acid regions, it is possible to identify the region that is recognized by the monoclonal antibody under investigation. Western blot analysis or ELISA is employed to determine if the specific monoclonal antibody under investigation is capable of binding one or more of the recombinant protein fragments. This approach
15 can ultimately identify the peptide regions that contains the epitope and, in some cases, to refine the epitope precisely to an 8-11 amino acid sequence.

Construct design and creation

The first step in epitope mapping is the design of nested gene truncations.
20 Frequently, the gene is divided into four equal parts for further analysis.

Gene cloning strategy

The general cloning strategy begins with PCR-based generation of the cloned gene fragments. In order to efficiently express the cloned fragment, especially when
25 using small amino acid regions, the cloned fragment is expressed as a fusion protein, i.e. fused to another carrier protein that is stably expressed in the system. Green fluorescent protein (GFP) is frequently used as the carrier protein. GFP is included as a fusion partner to stabilize the truncation fragments and improve expression during the subsequent *in vitro* protein expression step. GFP also permits the tracking of
30 fusion-protein expression using anti-GFP antibodies.

Cloning to create the GFP-protein construct is performed using either the mega-priming approach or through the use of plasmid cloning into the pScreen-GFP vector. Generally, the truncation fragments are fused to GFP and control sequences necessary for protein expression using a technique called megapriming.

Megapriming is the joining of two or more DNA fragments by annealing homologous regions at the end of the respective fragments and extending the annealed single-stranded DNA with a thermostable DNA polymerase. This process creates one large DNA fragment from two or more smaller fragments, linking them by their shared sequence. This large fragment is then amplified using standard PCR.

If megapriming cannot be used successfully, the truncation fragments can be cloned into a plasmid containing GFP and protein-expression control sequences. This cloning creates the GFP/fragment fusions necessary for epitope mapping. The remainder of the protocol can then proceed as described below.

Protein expression

The expression constructs created by, for example, megapriming are then introduced into the Rapid Translation System (RTS). RTS is a cell-free protein expression system derived from *E. coli* lysates. This system permits rapid (3-4 hour) expression of proteins from DNA templates.

If RTS does not produce adequate levels of protein expression, then the truncation fragments will be cloned into the GFP protein-expression plasmid. These fusion plasmids are then transformed into an *E. coli* strain optimized for protein expression. Protein expression is induced in a growing culture of bacteria and, following outgrowth, the cells are lysed. The proteins in the complex cell lysate are then separated by polyacrylamide gel electrophoresis (PAGE), and the remainder of the protocol is the same as below.

Protein detection and epitope mapping

Protein fragments produced by RTS are separated using PAGE and transferred onto nitrocellulose membranes. The membrane-bound proteins are then exposed to the antibody under investigation in solution. Antibody/protein binding is identified using colorimetric techniques known in the art.

Antibody binding of the full-length protein and some subset of the truncated protein fragments constitutes a positive result. If the absence of a particular section of the protein eliminates antibody binding, then the epitope lies on this fragment.

If the antibody to be mapped does not recognize protein bound to nitrocellulose membranes, then alternative methods for detecting antibody/protein

interactions, such as, for example, ELISA or immunoprecipitation are used. Methods for detecting antibody/protein interactions are well known in the art.

Refining the epitope location

5 Since the above-described protocol will only narrow the location of the epitope down to approximately one-quarter of the protein, it is necessary to repeat the process on the quarter of the protein determined to contain the epitope in order to further resolve the location of the epitope. For a very large protein, it may be necessary to repeat this process two to three times to narrow the epitope down to 8-15
10 amino acids.

Example 4: Characterization of Epitopes for MCM2 Monoclonal Antibodies 27C5.6 and 26H6.19

Epitope mapping for MCM2 Monoclonal Antibodies 27C5.6 and 26H6.19 was
15 carried out essentially as described in Example 3. Specifically, PCR was used to create MCM2 gene truncations, followed by RTS to generate recombinant MCM2 protein fragments, and finally western blotting to detect antibody binding to MCM2. GFP was joined with the MCM2 gene truncations in a second round of PCR to ensure robust and stable expression in RTS.

20 The full-length coding sequence for MCM2 (SEQ ID NO:2; NM_004526) has a size of 2715 bp. However, the cDNA that was used to express the recombinant MCM2 protein and that was used to immunize mice during the production of MCM2 antibodies had a gene size of 2688 bp (SEQ ID NO:5). The truncated MCM2 cDNA used had a 27 bp region missing at the 5' end of the MCM2 protein, specifically the
25 fragment ATGGCGGAATCATCGGAATCCTTCACC (SEQ ID NO:6). The following sequential steps were carried out in order to epitope map the MCM2-27C5.6 antibody:

 Since the MCM2 gene was large (>1000bp) and to minimize the number of iterations of PCR needed, the gene was equally divided into six regions [1-6] of
30 approximately 400 bp. Overlapping sequences, which contain homologous sequence to permit mega priming during a second PCR cycle and restriction sites for a second option of sub-cloning into pScreen-GFP plasmid, were added to the gene of interest during the first PCR. The first round of PCR created fragments of the truncated MCM2 nucleotide sequence (SEQ ID NO:5) including: region [1] was 1-426 bp,

region [1-2] was 1-888 bp, region [1-3] was 1-1377 bp, region [1-4] was 1-1845 bp, region [1-5] was 1-2241 bp, region [1-6] was 1-2688 bp, and finally region [2-6] was 427 -2688 bp. Individual regions (example region [5]) were not expressed to avoid missing epitopes that were present in junction sequence between regions.

5 The first round PCR products of MCM2 were subcloned into pSCREEN-GFP (BamH1-Xho1), as the fragment sizes were too large for mega-priming. The only truncation that was unsuccessful was the full length region [1-6]. The original primers used to amplify the full-length gene and truncations were engineered to include restriction sites (5' end BAMH1; 3'end XHO1) to allow direct subcloning
10 into pSCREEN-GFP.

 The GFP-gene fusions created were used as a template for protein production in the RTS reaction using the RTS 100 *E. coli* HY kit from Roche. The protein products from RTS were acetone precipitated, loaded directly onto a denaturing polyacrylimide gel, and analyzed by western blotting. The western blot was probed
15 directly with the 27C5.6 monoclonal antibody and GFP antibodies.

 The first round of RTS products were probed with both GFP antibodies and the MCM2 monoclonal antibody 27C5.6. A positive band was detected in region [1-3]. The above process was repeated using the fragment encompassed by region [1-3] as the starting sequence.

20 A second round of RTS produced a positive result for the 27C5.6 antibody in the region MCM2-3Q3 (CQSAGPFEVNMEETIYQNYQRIRIQESP (SEQ ID NO:7); corresponding to amino acid residues 355 to 382 of SEQ ID NO:1). The above process was repeated using the fragment encompassed by region MCM2-3Q3 as the starting sequence.

25 A third round of RTS produced a positive result for the 27C5.6 antibody in the region MCM2-3Q3.2 (IYQNYQRIRIQESP (SEQ ID NO:3); corresponding to amino acid residues 369 to 382 of SEQ ID NO:1). No positive result was obtained in region MCM2-3Q3.1 (CQSAGPFEVNMEET (SEQ ID NO:8); corresponding to amino acid residues 355 to 368 of SEQ ID NO:1) or in MCM2-3Q3.2 (EVNMEETIYQNYQR
30 (SEQ ID NO:9); corresponding to amino acid residues 362 to 375 of SEQ ID NO:1).

Results

 Initial results showed that the epitope for the MCM2 monoclonal antibody 27C5.6 is located within the N-terminal region of the MCM2 protein. Continued

truncations of the MCM2 protein showed that the epitope recognized by 27C5.6 is located within a fourteen amino acid region, specifically corresponding to amino acid residues 369-382 of SEQ ID NO:1 (IYQNYQRIRIQESP (SEQ ID NO:3)).

Additional rounds of RTS may be able to refine the epitope location further.

- 5 The identical process described above was used to identify the epitope for MCM2 monoclonal antibody 26H6.19. Initial results indicated that the epitope was located within the C-terminal region of the MCM2 protein. The epitope was preliminarily defined to a twenty-three amino acid region, specifically corresponding to amino acid residues 688-710 of SEQ ID NO:1
- 10 (PSNKEEEGLANGSAAEPAMPNTY (SEQ ID NO:4)). Further analysis refined the epitope of MCM2 monoclonal antibody 26H6.19 to a ten amino acid region comprising amino acid residues 683-692 of SEQ ID NO:1 (HVRHHPSNKE (SEQ ID NO:14)).

THAT WHICH IS CLAIMED:

1. A monoclonal antibody that is capable of specifically binding to MCM2, or a variant or fragment thereof, wherein the antibody is selected from the group consisting of:
- 5
- (a) the monoclonal antibody produced by the hybridoma cell line 27C5.6, deposited with the ATCC as Patent Deposit No. PTA-6668;
 - (b) the monoclonal antibody produced by the hybridoma cell line 26H6.19, deposited with the ATCC as Patent Deposit No. PTA-6667;
 - 10 (c) a monoclonal antibody having the binding characteristics of the monoclonal antibody produced by the hybridoma cell line 27C5.6 or 26H6.19;
 - (d) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 27C5.6 or 26H6.19;
 - 15 (e) a monoclonal antibody that binds to an epitope comprising the amino acid sequence set forth in SEQ ID NO:3;
 - (f) a monoclonal antibody that binds to an epitope comprising the amino acid sequence of SEQ ID NO:14
 - (g) a monoclonal antibody that competes in a competitive binding assay with the monoclonal antibody produced by the hybridoma cell line 27C5.6 or 20 26H6.19; and,
 - (h) a monoclonal antibody that is an antigen binding fragment of a monoclonal antibody of (a) – (g), wherein the fragment retains the capability of specifically binding to MCM2, or a variant or fragment thereof.
- 25
2. The hybridoma cell line 27C5.6, deposited with the ATCC as Patent Deposit No. PTA-6668.
3. The hybridoma cell line 26H6.19, deposited with the ATCC as Patent Deposit No. PTA-6667.
- 30
4. A hybridoma cell line capable of producing a monoclonal antibody of claim 1.

5. A kit for diagnosing high-grade cervical disease comprising at least one monoclonal antibody according to claim 1.

6. The kit of claim 5, wherein the monoclonal antibody is the monoclonal antibody produced by the hybridoma cell line 27C5.6, deposited with the ATCC as Patent Deposit No. PTA-6668, or the monoclonal antibody produced by the hybridoma cell line 26H6.19, deposited with the ATCC as Patent Deposit No. PTA-6667.

7. The kit of claim 5 comprising at least two antibodies, wherein a first antibody is the monoclonal antibody produced by the hybridoma cell line 27C5.6, deposited with the ATCC as Patent Deposit No. PTA-6668, and a second antibody is the monoclonal antibody produced by the hybridoma cell line 26H6.19, deposited with the ATCC as Patent Deposit No. PTA-6667.

8. The kit of claim 7 further comprising an antibody that specifically binds to Topo2A.

9. The kit of claim 7, wherein each antibody is provided as a separate antibody reagent.

10. The kit of claim 7, wherein all of the antibodies are provided as an antibody cocktail.

11. The kit of claim 5, wherein said kit further comprises a peroxidase blocking reagent, a protein blocking reagent, chemicals for the detection of antibody binding to said biomarker proteins, a counterstain, a bluing agent, and instructions for use.

12. The kit of claim 5 further comprising reagents for Pap staining.

13. The kit of claim 12, wherein the reagents for Pap staining comprise EA50 and Orange G.

14. A method for diagnosing high-grade cervical disease in a patient, the method comprising:

- a) obtaining a cervical sample from the patient;
- b) contacting the sample with at least one monoclonal antibody according to claim 1 that specifically binds to MCM2; and,
- c) detecting binding of the antibody to MCM2.

15. The method of claim 14, wherein the monoclonal antibody is the monoclonal antibody produced by the hybridoma cell line 27C5.6, deposited with the ATCC as Patent Deposit No. PTA-6668, or the monoclonal antibody produced by the hybridoma cell line 26H6.19, deposited with the ATCC as Patent Deposit No. PTA-6667.

16. The method of claim 14 comprising contacting the sample with at least two monoclonal antibodies that specifically bind to MCM2, wherein a first antibody is the monoclonal antibody produced by the hybridoma cell line 27C5.6, deposited with the ATCC as Patent Deposit No. PTA-6668, and a second antibody is the monoclonal antibody produced by the hybridoma cell line 26H6.19, deposited with the ATCC as Patent Deposit No. PTA-6667.

17. The method of claim 16 further comprising contacting the sample with an antibody that specifically binds to Topo2A.

18. The method of claim 16, wherein the antibodies are contacted with the sample sequentially as individual antibody reagents or simultaneously as an antibody cocktail.

19. An isolated polypeptide comprising an epitope for binding an MCM2 monoclonal antibody, wherein the polypeptide comprises an amino acid sequence selected from the group consisting of:

- (a) a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:3 or 14; and,
- (b) a polypeptide having at least 90% sequence identity to SEQ ID NO:3 or 14, wherein the polypeptide has antigenic activity.

20. A method for producing an MCM2 antibody comprising immunizing an animal with a polypeptide according to claim 19.

- 5 21. A method for producing an MCM2 monoclonal antibody comprising:
- (a) immunizing an animal with a polypeptide according to claim 19 under conditions to elicit an immune response;
 - (b) isolating antibody-producing cells from the animal;
 - (c) fusing the antibody-producing cells with immortalized cells in
 - 10 culture to form monoclonal antibody-producing hybridoma cells;
 - (d) culturing the hybridoma cells; and,
 - (e) isolating monoclonal antibodies from culture.

SEQUENCE LISTING

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 Taylor, Adriann J.

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 THEIR USE IN THE DETECTION OF CERVICAL DISEASE

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	850 855 860	
70	atc cac aac ctc tct gca ttt tat gac agt gag ctc ttc agg atg aac	2640
	Ile His Asn Leu Ser Ala Phe Tyr Asp Ser Glu Leu Phe Arg Met Asn	
	865 870 875 880	

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aag ttc agc cac gac ctg aaa agg aaa atg atc ctg cag cag ttc tga 2688
Lys Phe Ser His Asp Leu Lys Arg Lys Met Ile Leu Gln Gln Phe *
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5

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10 <212> DNA
    <213> Artificial Sequence

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15     nucleotide sequence

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20 <210> 7
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    <213> Artificial Sequence

25 <220>
<223> MCM2 fragment corresponding to amino acid residues
    355 to 382 of SEQ ID NO:1

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    Gln Asn Tyr Gln Arg Ile Arg Ile Gln Glu Ser Pro
        20          25

35 <210> 8
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    <212> PRT
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40 <220>
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50 <210> 9
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    <212> PRT
    <213> Artificial Sequence

55 <220>
<223> MCM2 fragment corresponding to amino acid residues
    362 to 375 of SEQ ID NO:1

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60 Glu Val Asn Met Glu Glu Thr Ile Tyr Gln Asn Tyr Gln Arg

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5 <210> 11
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 <213> Artificial Sequence

10 <220>
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 immunogenic polypeptide

15 <223> Hexa-histidine tag

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 20 20 25 30
 Pro Gly Arg Asp Leu Pro Pro Phe Glu Asp Glu Ser Glu Gly Leu Leu
 35 40 45
 Gly Thr Glu Gly Pro Leu Glu Glu Glu Asp Gly Glu Glu Leu Ile
 50 55 60
 25 Gly Asp Gly Met Glu Arg Asp Tyr Arg Ala Ile Pro Glu Leu Asp Ala
 65 70 75 80
 Tyr Glu Ala Glu Gly Leu Ala Leu Asp Asp Glu Asp Val Glu Glu Leu
 85 90 95
 Thr Ala Ser Gln Arg Glu Ala Ala Glu Arg Ala Met Arg Gln Arg Asp
 100 105 110
 30 Arg Glu Ala Gly Arg Gly Leu Gly Arg Met Arg Arg Gly Leu Leu Tyr
 115 120 125
 Asp Ser Asp Glu Glu Asp Glu Glu Arg Pro Ala Arg Lys Arg Arg Gln
 130 135 140
 35 Val Glu Arg Ala Thr Glu Asp Gly Glu Glu Asp Glu Glu Met Ile Glu
 145 150 155 160
 Ser Ile Glu Asn Leu Glu Asp Leu Lys Gly His Ser Val Arg Glu Trp
 165 170 175
 Val Ser Met Ala Gly Pro Arg Leu Glu Ile His His Arg Phe Lys Asn
 180 185 190
 40 Phe Leu Arg Thr His Val Asp Ser His Gly His Asn Val Phe Lys Glu
 195 200 205
 Arg Ile Ser Asp Met Cys Lys Glu Asn Arg Glu Ser Leu Val Val Asn
 210 215 220
 45 Tyr Glu Asp Leu Ala Ala Arg Glu His Val Leu Ala Tyr Phe Leu Pro
 225 230 235 240
 Glu Ala Pro Ala Glu Leu Leu Gln Ile Phe Asp Glu Ala Ala Leu Glu
 245 250 255
 Val Val Leu Ala Met Tyr Pro Lys Tyr Asp Arg Ile Thr Asn His Ile
 260 265 270
 50 His Val Arg Ile Ser His Leu Pro Leu Val Glu Glu Leu Arg Ser Leu
 275 280 285
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 290 295 300
 55 Ser Cys Thr Gly Val Leu Pro Gln Leu Ser Met Val Lys Tyr Asn Cys
 305 310 315 320
 Asn Lys Cys Asn Phe Val Leu Gly Pro Phe Cys Gln Ser Gln Asn Gln
 325 330 335
 Glu Val Lys Pro Gly Ser Cys Pro Glu Cys Gln Ser Ala Gly Pro Phe
 340 345 350
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	Glu	Val	Asn	Met	Glu	Glu	Thr	Ile	Tyr	Gln	Asn	Tyr	Gln	Arg	Ile	Arg
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	Ile	Gln	Glu	Ser	Pro	Gly	Lys	Val	Ala	Ala	Gly	Arg	Leu	Pro	Arg	Ser
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5	Lys	Asp	Ala	Ile	Leu	Leu	Ala	Asp	Leu	Val	Asp	Ser	Cys	Lys	Pro	Gly
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	Asp	Glu	Ile	Glu	Leu	Thr	Gly	Ile	Tyr	His	Asn	Asn	Tyr	Asp	Gly	Ser
					405					410					415	
10	Leu	Asn	Thr	Ala	Asn	Gly	Phe	Pro	Val	Phe	Ala	Thr	Val	Ile	Leu	Ala
				420					425					430		
	Asn	His	Val	Ala	Lys	Lys	Asp	Asn	Lys	Val	Ala	Val	Gly	Glu	Leu	Thr
			435					440					445			
	Asp	Glu	Asp	Val	Lys	Met	Ile	Thr	Ser	Leu	Ser	Lys	Asp	Gln	Gln	Ile
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15	Gly	Glu	Lys	Ile	Phe	Ala	Ser	Ile	Ala	Pro	Ser	Ile	Tyr	Gly	His	Glu
		465			470						475					480
	Asp	Ile	Lys	Arg	Gly	Leu	Ala	Leu	Ala	Leu	Phe	Gly	Gly	Glu	Pro	Lys
					485				490						495	
20	Asn	Pro	Gly	Gly	Lys	His	Lys	Val	Arg	Gly	Asp	Ile	Asn	Val	Leu	Leu
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	Cys	Gly	Asp	Pro	Gly	Thr	Ala	Lys	Ser	Gln	Phe	Leu	Lys	Tyr	Ile	Glu
			515					520					525			
	Lys	Val	Ser	Ser	Arg	Ala	Ile	Phe	Thr	Thr	Gly	Gln	Gly	Ala	Ser	Ala
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25	Val	Gly	Leu	Thr	Ala	Tyr	Val	Gln	Arg	His	Pro	Val	Ser	Arg	Glu	Trp
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30	Ile	Asp	Glu	Phe	Asp	Lys	Met	Asn	Asp	Gln	Asp	Arg	Thr	Ser	Ile	His
				580					585					590		
	Glu	Ala	Met	Glu	Gln	Gln	Ser	Ile	Ser	Ile	Ser	Lys	Ala	Gly	Ile	Val
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		610					615					620				
35	Gly	Gly	Arg	Tyr	Asp	Pro	Ser	Leu	Thr	Phe	Ser	Glu	Asn	Val	Asp	Leu
					625		630				635					640
	Thr	Glu	Pro	Ile	Ile	Ser	Arg	Phe	Asp	Ile	Leu	Cys	Val	Val	Arg	Asp
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40	Thr	Val	Asp	Pro	Val	Gln	Asp	Glu	Met	Leu	Ala	Arg	Phe	Val	Val	Gly
				660					665					670		
	Ser	His	Val	Arg	His	His	Pro	Ser	Asn	Lys	Glu	Glu	Glu	Gly	Leu	Ala
			675					680					685			
	Asn	Gly	Ser	Ala	Ala	Glu	Pro	Ala	Met	Pro	Asn	Thr	Tyr	Gly	Val	Glu
		690				695						700				
45	Pro	Leu	Pro	Gln	Glu	Val	Leu	Lys	Lys	Tyr	Ile	Ile	Tyr	Ala	Lys	Glu
					705		710				715					720
	Arg	Val	His	Pro	Lys	Leu	Asn	Gln	Met	Asp	Gln	Asp	Lys	Val	Ala	Lys
					725					730					735	
50	Met	Tyr	Ser	Asp	Leu	Arg	Lys	Glu	Ser	Met	Ala	Thr	Gly	Ser	Ile	Pro
				740					745					750		
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			755					760						765		
	Ala	Arg	Ile	His	Leu	Arg	Asp	Tyr	Val	Ile	Glu	Asp	Asp	Val	Asn	Met
			770				775					780				
55	Ala	Ile	Arg	Val	Met	Leu	Glu	Ser	Phe	Ile	Asp	Thr	Gln	Lys	Phe	Ser
					785		790				795					800
	Val	Met	Arg	Ser	Met	Arg	Lys	Thr	Phe	Ala	Arg	Tyr	Leu	Ser	Phe	Arg
					805					810					815	
60	Arg	Asp	Asn	Asn	Glu	Leu	Leu	Leu	Phe	Ile	Leu	Lys	Gln	Leu	Val	Ala
				820						825					830	

Glu Gln Val Thr Tyr Gln Arg Asn Arg Phe Gly Ala Gln Gln Asp Thr
 835 840 845
 Ile Glu Val Pro Glu Lys Asp Leu Val Asp Lys Ala Arg Gln Ile Asn
 850 855 860
 5 Ile His Asn Leu Ser Ala Phe Tyr Asp Ser Glu Leu Phe Arg Met Asn
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 Lys Phe Ser His Asp Leu Lys Arg Lys Met Ile Leu Gln Gln Phe Leu
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 10 Glu Gly Gly His His His His His
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 15 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Nucleotide sequence encoding the MCM2-FLAG
 20 polypeptide

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    <211> 905
    <212> PRT
    <213> Artificial Sequence

15 <220>
    <223> Amino acid sequence for the MCM2-FLAG polypeptide

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25 Gly Thr Glu Gly Pro Leu Glu Glu Glu Glu Asp Gly Glu Glu Leu Ile
    50 55 60
    Gly Asp Gly Met Glu Arg Asp Tyr Arg Ala Ile Pro Glu Leu Asp Ala
    65 70 75 80
30 Tyr Glu Ala Glu Gly Leu Ala Leu Asp Asp Glu Asp Val Glu Glu Leu
    85 90 95
    Thr Ala Ser Gln Arg Glu Ala Ala Glu Arg Ala Met Arg Gln Arg Asp
    100 105 110
    Arg Glu Ala Gly Arg Gly Leu Gly Arg Met Arg Arg Gly Leu Leu Tyr
    115 120 125
35 Asp Ser Asp Glu Glu Asp Glu Glu Arg Pro Ala Arg Lys Arg Arg Gln
    130 135 140
    Val Glu Arg Ala Thr Glu Asp Gly Glu Glu Asp Glu Glu Met Ile Glu
    145 150 155 160
40 Ser Ile Glu Asn Leu Glu Asp Leu Lys Gly His Ser Val Arg Glu Trp
    165 170 175
    Val Ser Met Ala Gly Pro Arg Leu Glu Ile His His Arg Phe Lys Asn
    180 185 190
    Phe Leu Arg Thr His Val Asp Ser His Gly His Asn Val Phe Lys Glu
    195 200 205
45 Arg Ile Ser Asp Met Cys Lys Glu Asn Arg Glu Ser Leu Val Val Asn
    210 215 220
    Tyr Glu Asp Leu Ala Ala Arg Glu His Val Leu Ala Tyr Phe Leu Pro
    225 230 235 240
50 Glu Ala Pro Ala Glu Leu Leu Gln Ile Phe Asp Glu Ala Ala Leu Glu
    245 250 255
    Val Val Leu Ala Met Tyr Pro Lys Tyr Asp Arg Ile Thr Asn His Ile
    260 265 270
    His Val Arg Ile Ser His Leu Pro Leu Val Glu Glu Leu Arg Ser Leu
    275 280 285
55 Arg Gln Leu His Leu Asn Gln Leu Ile Arg Thr Ser Gly Val Val Thr
    290 295 300
    Ser Cys Thr Gly Val Leu Pro Gln Leu Ser Met Val Lys Tyr Asn Cys
    305 310 315 320
60 Asn Lys Cys Asn Phe Val Leu Gly Pro Phe Cys Gln Ser Gln Asn Gln
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			370				375					380				
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						390					395					400
10	Asp	Glu	Ile	Glu	Leu	Thr	Gly	Ile	Tyr	His	Asn	Asn	Tyr	Asp	Gly	Ser
					405					410					415	
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				420					425					430		
	Asn	His	Val	Ala	Lys	Lys	Asp	Asn	Lys	Val	Ala	Val	Gly	Glu	Leu	Thr
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15	Asp	Glu	Asp	Val	Lys	Met	Ile	Thr	Ser	Leu	Ser	Lys	Asp	Gln	Gln	Ile
							455					460				
	Gly	Glu	Lys	Ile	Phe	Ala	Ser	Ile	Ala	Pro	Ser	Ile	Tyr	Gly	His	Glu
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20	Asp	Ile	Lys	Arg	Gly	Leu	Ala	Leu	Ala	Leu	Phe	Gly	Gly	Glu	Pro	Lys
					485					490					495	
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	Cys	Gly	Asp	Pro	Gly	Thr	Ala	Lys	Ser	Gln	Phe	Leu	Lys	Tyr	Ile	Glu
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25	Lys	Val	Ser	Ser	Arg	Ala	Ile	Phe	Thr	Thr	Gly	Gln	Gly	Ala	Ser	Ala
							535					540				
	Val	Gly	Leu	Thr	Ala	Tyr	Val	Gln	Arg	His	Pro	Val	Ser	Arg	Glu	Trp
						550					555					560
30	Thr	Leu	Glu	Ala	Gly	Ala	Leu	Val	Leu	Ala	Asp	Arg	Gly	Val	Cys	Leu
					565					570					575	
	Ile	Asp	Glu	Phe	Asp	Lys	Met	Asn	Asp	Gln	Asp	Arg	Thr	Ser	Ile	His
				580					585					590		
	Glu	Ala	Met	Glu	Gln	Gln	Ser	Ile	Ser	Ile	Ser	Lys	Ala	Gly	Ile	Val
				595				600					605			
35	Thr	Ser	Leu	Gln	Ala	Arg	Cys	Thr	Val	Ile	Ala	Ala	Ala	Asn	Pro	Ile
							615					620				
	Gly	Gly	Arg	Tyr	Asp	Pro	Ser	Leu	Thr	Phe	Ser	Glu	Asn	Val	Asp	Leu
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40	Thr	Glu	Pro	Ile	Ile	Ser	Arg	Phe	Asp	Ile	Leu	Cys	Val	Val	Arg	Asp
					645					650					655	
	Thr	Val	Asp	Pro	Val	Gln	Asp	Glu	Met	Leu	Ala	Arg	Phe	Val	Val	Gly
				660					665					670		
	Ser	His	Val	Arg	His	His	Pro	Ser	Asn	Lys	Glu	Glu	Glu	Gly	Leu	Ala
				675				680					685			
45	Asn	Gly	Ser	Ala	Ala	Glu	Pro	Ala	Met	Pro	Asn	Thr	Tyr	Gly	Val	Glu
							695					700				
	Pro	Leu	Pro	Gln	Glu	Val	Leu	Lys	Lys	Tyr	Ile	Ile	Tyr	Ala	Lys	Glu
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50	Arg	Val	His	Pro	Lys	Leu	Asn	Gln	Met	Asp	Gln	Asp	Lys	Val	Ala	Lys
					725					730					735	
	Met	Tyr	Ser	Asp	Leu	Arg	Lys	Glu	Ser	Met	Ala	Thr	Gly	Ser	Ile	Pro
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	Ile	Thr	Val	Arg	His	Ile	Glu	Ser	Met	Ile	Arg	Met	Ala	Glu	Ala	His
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55	Ala	Arg	Ile	His	Leu	Arg	Asp	Tyr	Val	Ile	Glu	Asp	Asp	Val	Asn	Met
							775					780				
	Ala	Ile	Arg	Val	Met	Leu	Glu	Ser	Phe	Ile	Asp	Thr	Gln	Lys	Phe	Ser
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60	Val	Met	Arg	Ser	Met	Arg	Lys	Thr	Phe	Ala	Arg	Tyr	Leu	Ser	Phe	Arg
					805					810					815	

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Arg Asp Asn Asn Glu Leu Leu Leu Phe Ile Leu Lys Gln Leu Val Ala
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Glu Gln Val Thr Tyr Gln Arg Asn Arg Phe Gly Ala Gln Gln Asp Thr
      835      840      845
5  Ile Glu Val Pro Glu Lys Asp Leu Val Asp Lys Ala Arg Gln Ile Asn
      850      855      860
Ile His Asn Leu Ser Ala Phe Tyr Asp Ser Glu Leu Phe Arg Met Asn
865      870      875
10 Lys Phe Ser His Asp Leu Lys Arg Lys Met Ile Leu Gln Gln Phe Leu
      885      890      895
Glu Asp Tyr Lys Asp Asp Asp Asp Lys
      900      905

15 <210> 14
    <211> 10
    <212> PRT
    <213> Artificial Sequence

20 <220>
    <223> Amino acid sequence for the MCM2 epitope of
          monoclonal antibody 26H6.19 (refined)

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25 His Val Arg His His Pro Ser Asn Lys Glu
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