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(54) Title: CALIBRATOR QUALITY CONTROL CELL DEVICE FOR IMMUNOHISTOCHEMISTRY ASSAY AND METHODS OF USE THEREOF

(57) Abstract: This disclosure relates to immunohistochemistry, and specifically to a quality control device for an immunohistochemistry assay and methods of use thereof. More specifically, disclosed herein is a calibrator cell pellet and methods of use as a control in an immunohistochemistry assay. The calibrator cell pellets are used, in some embodiments, as standards for quantifying the intensity of immunohistochemical staining, for instance for normalizing immunohistochemical staining to a calibrated standard, thereby allowing more accurate quantitation of staining intensity.

CALIBRATOR QUALITY CONTROL CELL DEVICE FOR IMMUNOHISTOCHEMISTRY ASSAY AND METHODS OF USE THEREOF

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of the earlier filing date of U.S. Provisional Application Nos. 61/009,426, 61/009,420 and 61/009,360 filed on December 28, 2007, each of which is incorporated herein by reference in its entirety.

FIELD OF THE DISCLOSURE

This disclosure relates to immunohistochemistry, and specifically to methods of using a quality control device for an immunohistochemistry assay.

BACKGROUND

Immunohistochemistry (IHC) is a technique involving the use of specific binding agents, such as antibodies, to detect specific antigens that may be present in a tissue sample. IHC is widely used in clinical and diagnostic applications, such as to diagnose particular disease states or conditions. For example, a diagnosis of a particular type of cancer can be made based on the presence of a particular marker molecule present in a sample obtained from a subject. IHC is also widely used in basic research to understand the distribution and localization of biomarkers in different parts of a tissue.

A deficiency of IHC is its lack of standards, making accurate quantification of the results difficult, if not impossible. The staining results achieved by IHC typically are scored subjectively, for instance on a four-point scale with 0 being negative and 4 being intensely positive. Because there are no standards for comparison, IHC is not quantitative, and there are no methods to correct for intra-run variability (the difference in staining between samples stained at the same time under similar conditions) and inter-run variability (the variability between slides that were stained at different times and possibly under different conditions). Thus, a number

of potential errors can be introduced into the staining procedure, leading to erroneous results.

Variability in IHC staining is well recognized and universal. Attempts to minimize variability require stringent adherence to the protocol, the proper use of controls, and automation. Even with these precautions, a degree of variability inevitably occurs and is accepted as inherent to the method. Thus, it would be desirable to have methods for controlling for both intra- and inter-run variability, as well as to permit quantification of staining intensity.

SUMMARY OF THE DISCLOSURE

Disclosed herein are quality control devices for IHC assays and methods of using them. One embodiment of the method includes (i) providing a first universal positive control cell pellet section and a first test sample on same solid support, wherein the first universal positive control cell pellet section contains a known amount of a known antigen; (ii) performing immunohistochemistry on the first universal positive control cell pellet section and the first test sample; (iii) analyzing immunohistochemical staining intensity of the first universal positive control cell pellet section and the first test sample; (iv) assigning a numerical value to the immunohistochemical staining intensity of the first universal positive control cell pellet section; (v) assigning a numerical value to the immunohistochemical staining intensity of the first test sample; (vi) comparing the immunohistochemical staining intensity of the first universal positive control cell pellet section to an expected value for the first universal positive control cell pellet section, thereby providing a deviation between the immunohistochemical staining of the first universal positive control cell pellet section and the expected value; (vii) calculating a correction factor based on the deviation between the immunohistochemical staining of the first universal positive control cell pellet section and the expected value; and (viii) using the correction factor to normalize the numerical value assigned to the immunohistochemical staining intensity of the first test sample to the expected value for the first universal positive control cell pellet section.

Kits for performing IHC are also provided. For example, the kit can include

one or more of the disclosed quality control devices. In one example, the kit includes a slide that includes a universal cell pellet control section, instructions for carrying out the method, and/or at least one of a primary antibody and a secondary antibody.

The foregoing and other features of the disclosure will become more apparent from the following detailed description of a several embodiments which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a digital image of a quality control sample treated with a mouse monoclonal antibody to vimentin antigen illustrating a positive IHC reaction.

FIG. 2 illustrates a microscopic (10x) view of a test sample after IHC staining with vimentin antigen.

FIG. 3 shows a microscopic (40x) view of a universal positive control cell sample treated with a mouse monoclonal antibody to vimentin antigen in which the brown colored reaction illustrates positive staining.

FIG. 4 is a schematic diagram of a test tissue, a universal positive control cell pellet section, an antigen-retrieval control cell pellet section, and an endogenous peroxidase control cell pellet section placed on a solid support.

FIG. 5 provides a series of digital images and histograms illustrating staining variability in calibrator cells. The upper left panel illustrates under-staining of calibrator cells, the upper center panel optimal staining and the upper right panel over-staining. Histograms beneath each panel illustrate the calculated staining for the calibrator cells.

FIG. 6 provides a series of digital images and histograms illustrating staining variability in test specimens. The upper left panel illustrates under-staining, the upper center panel optimal staining and the upper right panel over-staining. Histograms beneath each panel illustrate the calculated staining for the calibrator cells.

FIG. 7 provides a series of digital images prior to (upper row) and following (lower row) removal of variability.

FIG. 8 provides a series of histograms representing calculated staining intensity of three test specimens after correction.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

I. Abbreviations and Terms

DAB:	diaminobenzidine
ELISA:	Enzyme-linked immunosorbent assay
HRP:	Horseradish peroxidase
IHC:	immunohistochemistry
scFv:	single chain Fv proteins
dsFv:	disulfide stabilized Fv proteins
VH:	variable heavy
VL:	variable light

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes VII*, published by Oxford University Press, 2000 (ISBN 019879276X); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Publishers, 1994 (ISBN 0632021829); Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by Wiley, John & Sons, Inc., 1995 (ISBN 0471186341); and George P. Rédei, *Encyclopedic Dictionary of Genetics, Genomics, and Proteomics,* 2nd Edition, 2003 (ISBN: 0-471-26821-6). Definitions of common terms in IHC may be in Harlow and Lane, Antibodies: A Laboratory Manual, published by Cold Spring Harbor Laboratory Press; 1st Edition, 1988 (ISBN-10: 0879693142).

The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. The singular forms "a," "an," and "the" refer to one or more than one, unless the context clearly dictates otherwise. For example, the

term "comprising an antibody" includes one or more antibodies and is considered equivalent to the phrase "comprising at least one antibody." The term "or" refers to a single element of stated alternative elements or a combination of two or more elements, unless the context clearly indicates otherwise. As used herein, "comprises" means "includes." Thus, "comprising A or B," means "including A, B, or A and B," without excluding additional elements.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

To facilitate review of the various embodiments of this disclosure, the following explanations of specific terms are provided:

Antibody: A polypeptide that includes at least a light chain or heavy chain immunoglobulin variable region and specifically binds an epitope of an antigen. Antibodies include monoclonal antibodies, polyclonal antibodies, or fragments of antibodies as well as others known in the art. In some examples, an antibody is labeled with a detectable label such as an enzyme of fluorophore.

The term "specifically binds" refers to, with respect to an antigen, the preferential association of an antibody or other ligand, in whole or part, with a specific polypeptide. A specific binding agent binds substantially only to a defined target. It is recognized that a minor degree of non-specific interaction may occur between a molecule, such as a specific binding agent, and a non-target polypeptide. Nevertheless, specific binding can be distinguished as mediated through specific recognition of the antigen. Although selectively reactive antibodies bind antigen, they can do so with low affinity. Specific binding typically results in greater than 2-fold, such as greater than 5-fold, greater than 10-fold, or greater than 100-fold increase in amount of bound antibody or other ligand (per unit time) to a target polypeptide, such as compared to a non-target polypeptide. A variety of immunoassay formats are appropriate for selecting antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically

immunoreactive with a protein. See Harlow & Lane, Antibodies, *A Laboratory Manual*, Cold Spring Harbor Publications, New York (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

Antibodies are composed of a heavy and a light chain, each of which has a variable region, termed the variable heavy (VH) region and the variable light (VL) region. Together, the VH region and the VL region are responsible for binding the antigen recognized by the antibody. This includes intact immunoglobulins and the variants and portions of them well known in the art, such as Fab' fragments, F(ab)'2 fragments, single chain Fv proteins ("scFv"), and disulfide stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker, while in dsFvs, the chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. The term also includes recombinant forms such as chimeric antibodies (for example, humanized murine antibodies), heteroconjugate antibodies (such as, bispecific antibodies). See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, IL); Kuby, Immunology, 3rd Ed., W.H. Freeman & Co., New York, 1997.

A "monoclonal antibody" is an antibody produced by a single clone of B-lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. These fused cells and their progeny are termed "hybridomas." Monoclonal antibodies include humanized monoclonal antibodies.

Antigen: A molecule that stimulates an immune response. Antigens are usually proteins or polysaccharides. An epitope is an antigenic determinant. These are particular chemical groups or peptide sequences on a molecule that are antigenic, such that they elicit a specific immune response. An antibody binds a particular antigenic epitope. The binding of an antibody to a particular antigen or epitope of an antigen can be used to localize the position of the antigen for example in or on a

biological sample, or determine if the particular antigen is present in a biological sample. An **antigen of interest** is an antigen for which the IHC assay is designed to detect in a test sample. For example, to detect the **antigen of interest**, the primary antibody used in the IHC assay specifically binds to the **antigen of interest**.

Antigen retrieval: A process for recovering antigenicity in fixed processed tissue samples. Antigen retrieval is also sometimes referred to as epitope retrieval, target retrieval, or target unmasking. Various methods are used for **antigen retrieval**, including heat treatment, protease digestion, or a combination of heat and protease treatment.

Antigen-retrieval control: Refers to a cell pellet that serves as a positive control to confirm that an antigen-retrieval procedure was performed as part of the immunohistochemistry assay. In one example, the antigen-retrieval control cells express mouse immunoglobulin. Fixation of the cells renders mouse immunoglobulin undetectable by IHC (due to cross-linking of the protein) unless antigen retrieval is performed on the sample. Thus, a positive staining result with the antigen-retrieval control cell pellet indicates antigen retrieval was performed properly.

Antigen-specific control: Refers to a cell pellet in which the cells are known to express an antigen of interest such that when an IHC assay is performed, the antigen-specific control produces a positive signal.

Binding or stable binding: An association between two substances or molecules, such as the association of a specific binding agent (*e.g.*, antibody) with an antigen.

Binding affinity: The tendency of one molecule to bind (typically non-covalently) with another molecule, such as the tendency of one member of a specific binding pair to bind with another member of a specific binding pair. A binding affinity can be measured as a binding constant, which binding affinity for a specific binding pair (such as an antibody/antigen pair or nucleic acid probe/nucleic acid sequence pair) can be at least 1 x 10^5 M^{-1}, such as at least 1 x 10^6 M^{-1}, at least 1 x 10^7 M^{-1} or at least 1 x 10^8 M^{-1}. Binding affinity can calculated by a modification of the Scatchard method described by Frankel *et al.* (*Mol. Immunol.*, 16:101-106,

1979), or by an antigen/antibody dissociation rate. A high binding affinity can be measured by a competition radioimmunoassay. In several examples, a high binding affinity for an antibody/antigen pair is at least about 1 x 10^8 M^{-1}. In other embodiments, a high binding affinity is at least about 1.5 x 10^8 M^{-1}, at least about 2.0 x 10^8 M^{-1}, at least about 2.5 x 10^8 M^{-1}, at least about 3.0 x 10^8 M^{-1}, at least about 3.5 x 10^8 M^{-1}, at least about 4.0 x 10^8 M^{-1}, at least about 4.5 x 10^8 M^{-1}, or at least about 5.0 x 10^8 M^{-1}.

Carrier: A molecule to which a hapten or an antigen can be bound. Carrier molecules include immunogenic carriers and specific-binding carriers. When bound to an immunogenic carrier, the bound molecule may become immunogenic. Immunogenic carriers may be chosen to increase the immunogenicity of the bound molecule and/or to elicit antibodies against the carrier, which are diagnostically, analytically, and/or therapeutically beneficial. Covalent linking of a molecule to a carrier can confer enhanced immunogenicity and T-cell dependence (Pozsgay *et al.*, *PNAS* 96:5194-97, 1999; Lee *et al.*, *J. Immunol.* 116:1711-18, 1976; Dintzis *et al.*, *PNAS* 73:3671-75, 1976). Useful carriers include polymeric carriers, which can be natural (for example, proteins from bacteria or viruses), semi-synthetic or synthetic materials containing one or more functional groups to which a reactant moiety can be attached. Specific binding carriers can by any type of specific binding moiety, including an antibody, a nucleic acid, an avidin, a protein-nucleic acid.

Examples of suitable immunogenic carriers are those that can increase the immunogenicity of a hapten and/or help elicit antibodies against the hapten which are diagnostically, analytically, and/or therapeutically beneficial. Useful carriers include polymeric carriers, which can be natural (such as proteins like ovalbumin or keyhole limpet hemocyanin) or derived from a natural polymer isolated from any organism (including viruses), semi-synthetic or synthetic materials containing one or more functional groups, for example primary and/or secondary amino groups, azido groups, hydroxyl groups, or carboxyl groups, to which a reactant moiety can be attached. The carrier can be water soluble or insoluble, and can be a protein or polypeptide. Carriers that fulfill these criteria are generally known in the art (see, for example, Fattom *et al.*, *Infect. Immun.* 58:2309-12, 1990; Devi *et al.*, *PNAS*

88:7175-79, 1991; Szu *et al.*, *Infect. Immun.* 59:4555-61, 1991; Szu *et al.*, *J. Exp. Med.* 166:1510-24, 1987; and Pavliakova *et al.*, *Infect. Immun.* 68:2161-66, 2000).

Cell pellet: A collection of cells that has been compressed in volume to form a densely packed mass of cells. Cell pellets are typically formed by centrifugation of a collection of cells, such as cells suspended in growth medium.

Change: To become different in some way, for example to be altered, such as increased or decreased. A detectable change is one that can be detected, such as a change in the presence of an electromagnetic signal, such as chemical luminescence or fluorescence.

Conjugating, joining, bonding or linking: Covalently linking one molecule to another molecule, or to a cell or tissue. For example, conjugating, joining, bonding or linking includes making two polypeptides into one contiguous polypeptide molecule, or covalently attaching a hapten or other molecule to the surface of a cell. In one embodiment, the linkage is by chemical means. "Chemical means" refers to a reaction between the hapten and the cell such that there is a covalent bond formed.

Contacting: Placement in direct physical association, for example both in solid form and/or in liquid form (for example, the placement of a biological sample, such as a biological sample affixed to a slide, in contact with an antigen releasing solution).

Control: A sample or procedure performed to assess test validity. In one example, a control is a **quality control**, such as a **positive control**. For example, a positive control is a procedure or sample, such as a tissue or cell, that is similar to the actual test sample, but which is known from previous experience to give a positive result. The positive control confirms that the basic conditions of the test produce a positive result, even if none of the actual test samples produce such result. In a particular example, a positive control is a sample known by previous testing to contain the suspected antigen.

In other examples, a control is **a negative control**. A negative control is a procedure or test sample known from previous experience to give a negative result. The negative control demonstrates the base-line result obtained when a test does not

produce a measurable positive result; often the value of the negative control is treated as a "background" value to be subtracted from the test sample results. In a particular example, a negative control is a reagent that does not include the specific primary antibody. Other examples include **calibrator controls**, which are universal positive controls that contain a known amount of the universal positive control antigen. Such calibrator controls have an expected signal intensity, and therefore can be used to correct for inter- or intra-run staining variability.

Coupled: As used herein, an antigen "coupled" to a cell means the antigen is attached to the cell by chemical, physical or passive means. In one embodiment, the antigen is coupled to the cell by chemical conjugation. Methods of conjugation are well known in the art (see, for example, Hermanson, G.T., "Bioconjugate Techniques," Academic Press, San Diego, CA, 1996).

Covalently linked: Refers to a covalent linkage between atoms by the formation of a covalent bond characterized by the sharing of pairs of electrons between atoms. In one example, a detectable label is covalently linked to a specific binding agent, such as an antibody.

Detect: To determine if an agent (such as a signal or particular antigen or protein) is present or absent, for example, in a sample. In some examples, this can further include quantification.

Detectable Label: A detectable compound or composition that is attached directly or indirectly to another molecule, such as an antibody or a protein, to facilitate detection of that molecule. Specific, non-limiting examples of labels include fluorophores, enzymes, and radioactive isotopes.

Dispenser: A unit capable of dispensing or providing something. In one example, a dispenser is an object that supports or contains a device, such as a quality control device, and has a mechanism to detach or dispense the device. In some examples, a dispenser is configured to store multiple quality control devices and allow individual units of such devices to be efficiently dispensed, for example, by including a cutting mechanism (*e.g.*, a plurality of teeth) at one end of the device.

Endogenous antigen: An antigen synthesized within a cell. In one embodiment, the endogenous antigen is an immunoglobulin molecule.

Endogenous peroxidase control: Refers to a sample, such as a cell pellet, that serves as a control for endogenous peroxidase activity. If an IHC assay uses peroxidase as a means of assessing the presence of the antigen of interest, the test and control samples are blocked to eliminate endogenous peroxidase activity of the cells or tissue. A positive result indicates the sample was not properly blocked against endogenous peroxidase activity. A negative result indicates peroxidase activity was effectively blocked, thus the IHC assay is valid. Endogenous peroxidase control cells include cells having peroxidase activity, or pseudo-peroxidase activity. Such cells include, but are not limited to, cells of hematogenous origin, such as red blood cells.

Epitope: An antigenic determinant. These are particular chemical groups or contiguous or non-contiguous peptide sequences on a molecule that are antigenic, that is, that elicit a specific immune response. An antibody binds a particular antigenic epitope.

Exogenous antigen: An antigen coupled to the exterior of a cell. In one embodiment, the exogenous antigen is a hapten, such as fluorescein, biotin, dinitro phenol or digoxigenin.

Fixation: A process which preserves cells and tissue constituents in as close to a life-like state as possible and allows them to undergo preparative procedures without change. Fixation arrests the autolysis and bacterial decomposition processes which begin upon cell death, and stabilizes the cellular and tissue constituents so that they withstand the subsequent stages of tissue processing, such as for IHC.

Tissues may be fixed by either perfusion with or submersion in a **fixative,** such as an aldehyde (such as formaldehyde, paraformaldehyde, glutaraldehyde, and the like). Other fixatives include oxidizing agents (for example, metallic ions and complexes, such as osmium tetroxide and chromic acid), protein-denaturing agents (for example, acetic acid, methanol, and ethanol), fixatives of unknown mechanism (for example, mercuric chloride, acetone, and picric acid), combination reagents (for example, Carnoy's fixative, methacarn, Bouin's fluid, B5 fixative, Rossman's fluid, and Gendre's fluid), microwaves, and miscellaneous (for example, excluded volume fixation and vapour fixation). Additives may also be included in the fixative, such

as buffers, detergents, tannic acid, phenol, metal salts (for example, zinc chloride, zinc sulfate, and lithium salts), and lanthanum.

The most commonly used fixative in preparing samples for IHC is formaldehyde, generally in the form of a formalin solution (4% formaldehyde in a buffer solution, referred to as 10% buffered formalin).

Fluorophore: A chemical compound, which when excited by exposure to a particular stimulus, such as a defined wavelength of light, emits light (fluoresces), for example at a different wavelength (such as a longer wavelength of light).

Fluorophores are part of the larger class of luminescent compounds. Luminescent compounds include chemiluminescent molecules, which do not require a particular wavelength of light to luminesce, but rather use a chemical source of energy. Therefore, the use of chemiluminescent molecules (such as aequorin) can eliminate the need for an external source of electromagnetic radiation, such as a laser.

Examples of particular fluorophores that can be used in the methods disclosed herein are provided in U.S. Patent No. 5,866,366 to Nazarenko *et al.*, such as 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, anthranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumaran 151); cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5', 5"-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-

carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC (XRITC); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron™ Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives; LightCycler Red 640; Cy5.5; and Cy56-carboxyfluorescein; 5-carboxyfluorescein (5-FAM); boron dipyrromethene difluoride (BODIPY); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); acridine, stilbene, -6-carboxy-fluorescein (HEX), TET (Tetramethyl fluorescein), 6-carboxy-X-rhodamine (ROX), Texas Red, 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), Cy3, Cy5, VIC® (Applied Biosystems), LC Red 640, LC Red 705, Yakima yellow amongst others.

Other suitable fluorophores include those known to those skilled in the art, for example those available from Molecular Probes (Eugene, OR). In particular examples, a fluorophore is used as a donor fluorophore or as an acceptor fluorophore.

Hapten: A molecule, typically a small molecule that can combine specifically with an antibody, but typically is substantially incapable of being immunogenic except in combination with a carrier molecule. Examples of haptens include, but are not limited to fluorescein, biotin, dinitrophenol, and digoxigenin.

High throughput technique: Methods that allow rapid IHC staining of multiple samples, for example, through a combination of robotics, data processing

and control software, liquid handling devices, and sensitive detectors, high throughput techniques allows the rapid IHC staining of multiple samples.

Immunohistochemistry (IHC): A method of determining the presence or distribution of an antigen in a sample by detecting interaction of the antigen with a specific binding agent, such as an antibody.

Label: An agent capable of detection, for example by ELISA, spectrophotometry, flow cytometry, or microscopy. For example, a label can be attached to a specific binding agent, such as an antibody, thereby permitting detection of the specific binding agent and hence an antigen bound by the specific binding agent. Examples of labels include, but are not limited to, radioactive isotopes, enzyme substrates, co-factors, ligands, chemiluminescent agents, fluorophores (such as small molecule fluorophores or semiconductor nanocrystals), haptens, enzymes, and combinations thereof. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed for example in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989) and Ausubel *et al.* (*In Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1998).

Membrane: A support surface on which a sample can be immobilized. In some examples, a membrane is any surface that allows a sample, such as a quality control sample, to be immobilized and subsequently removed without significantly damaging the cellular components (*e.g.*, causing the sample to wrinkle, tear and the like) of the sample. In certain examples, a membrane includes a first surface that is a **detachable surface**. A detachable surface is any surface from which a sample can be detached from without significantly damaging the cellular components. Examples of a detachable surface include regenerated cellulose (*e.g.*, cellophane), paraffin, wax, and fluoropolymer coatings, such as polytetrafluoroethylene and perfluoroalkoxy. In some examples, the membrane is a membrane "strip" which includes multiple quality control samples, such as multiple control cell pellet sections. In certain examples, the membrane strip includes perforations assisting with the separation of individual quality control devices.

Polymeric substance: A substance composed of molecules with large molecular mass composed of repeating structural units, or monomers, connected by covalent chemical bonds. As used herein, examples of polymeric substances can include paraffin, agarose, and gelatin.

Quality control device: A device employed to assess assay validity, such as IHC assay validity. For example, a quality control device is a device useful for monitoring quality control of assays that measure analytes in cells or tissue samples obtained from biological samples. In some examples, a quality control device includes a membrane and a quality control sample, such as a universal positive control cell pellet section.

Sample: A sample, such as a biological sample, includes biological materials (such as nucleic acid and proteins, for example double-stranded nucleic acid binding proteins) obtained from an organism or a part thereof, such as a plant, animal, bacteria, and the like. In some examples, the biological sample is obtained from an animal subject, such as a human subject. A biological sample is any solid or fluid sample obtained from, excreted by or secreted by any living organism, including without limitation, single celled organisms, such as bacteria, yeast, protozoans, and amebas among others, multicellular organisms (such as plants or animals, including samples from a healthy or apparently healthy human subject or a human patient affected by a condition or disease to be diagnosed or investigated, such as cancer). For example, a biological sample can be a biological fluid obtained from, for example, blood, plasma, serum, urine, bile, ascites, saliva, cerebrospinal fluid, aqueous or vitreous humor, or any bodily secretion, a transudate, an exudate (for example, fluid obtained from an abscess or any other site of infection or inflammation), or fluid obtained from a joint (for example, a normal joint or a joint affected by disease). A biological sample can also be a sample obtained from any organ or tissue (including a biopsy or autopsy specimen, such as a tumor biopsy) or can include a cell (whether a primary cell or cultured cell) or medium conditioned by any cell, tissue or organ. In some examples, a biological sample is a nuclear extract. In some examples, a biological sample is bacterial cytoplasm. In certain examples, a sample is a quality control sample, such as one of the disclosed cell pellet section

samples. In other examples, a sample is a **test sample**. For example, a test sample is a cell, a tissue or cell pellet section prepared from a biological sample obtained from a subject. In an example, the subject is one that is at risk or has acquired a particular condition or disease.

Solid support: Any solid surface suitable for processing IHC samples, such as one or more of the disclosed control cell pellet samples and a test sample. In some examples, a solid support surface is a rigid, flat surface. For example, a solid support can be a rigid, flat, optically opaque and substantially non-fluorescent surface, such as a glass microscope slide. In additional examples, the solid support fits entirely onto the stage of a microscope and accommodates at least one quality control sample and test sample to allow simultaneous processing. In some examples, the solid support is any surface to which a sample can be permanently attached or affixed. For example, a solid support can include a non-detachable surface (*e.g.*, a surface to which a sample cannot be removed from without causing significant damage to the sample).

Tissue: A collection of interconnected cells that perform a similar function within an organism.

Universal positive control: A cell (or a cell pellet) that expresses a known endogenous antigen or includes a known exogenous antigen coupled to the cell surface. When used as a control in an IHC assay, the known antigen of the universal positive control is structurally unrelated to the antigen of interest, but always stains positive when the IHC procedure is performed correctly. In one embodiment, the known antigen is an immunoglobulin. In this case, a positive result with the universal positive control indicates that secondary antibody (*e.g.,* anti-immunoglobulin antibody) treatment and all subsequent steps of the IHC assay were performed correctly. In another embodiment, the known antigen is an exogenous antigen, such as fluorescein. In this case, a positive result with the universal positive control indicates that primary antibody (*e.g.,* anti-fluorescein antibody) treatment and all subsequent steps of the IHC assay were performed correctly.

II. Introduction

When using IHC, for example to analyze suspected disease tissue, typically the following procedures are performed: (1) isolate the desired tissue; (2) process the tissue in preparation for IHC analysis (which includes putting the tissue on a first microscope slide); (3) obtain another microscope slide containing a sample that can serve as a positive control, typically an antigen specific positive control; (4) stain both slides by IHC; and (5) examine both slides (*e.g.*, using a microscope). If the antigen is detected in the positive control sample, then the test is considered to be valid. If the target antigen (*e.g.*, antigen known to be a marker for a suspected disease) is detected in the test (*e.g.*, suspected disease) tissue, then the presence of the target antigen is confirmed (*e.g.*, confirming presence of the disease); if the test tissue is negative, the presence of the target antigen is not confirmed (*e.g.*, the disease is not confirmed).

A disadvantage of the positive control sample typically used in IHC is that the test sample is on one slide and the positive control sample is on another slide. Therefore, there are a number of potential errors that could be introduced into the staining procedure that would not be detected by the positive control sample. This often results in intra-run variability, such as one of the slides staining slightly stronger or slightly weaker than the other slide. Another source of error occurs when one test sample is compared to another test sample. Sometimes it is necessary to test multiple tissue samples simultaneously. In such instance, usually only a single positive control sample is run. Because of the known intra-run variability among samples, such comparisons are often not accurate.

One or more of these aforementioned limitations can be overcome by having a positive control sample on same microscope slide as the test sample. For example, a test sample which contains its own control on the same slide results in the test sample having its own internal control (*e.g.*, when the slide is stained, both the test sample and the positive control sample are stained at the same time under identical conditions). The reagents and the incubation times are identical. If the slide is over-stained or under-stained, the positive control sample will be similarly affected and any over- or under-staining will be immediately noticeable in the positive control

sample. By knowing that over- or under-staining has occurred, a variety of different corrective approaches are possible in order to correctly interpret the results.

However, if positive control samples are purchased from a commercial source, currently only positive control samples which are pre-attached to a microscope slide are commercially available. Therefore, if one has already prepared another slide with the test sample, then it is nearly impossible to physically combine the tissues and cells onto a single microscope slide.

The present disclosure solves one or more of the aforementioned problems by providing positive control cell pellet sections that are fixed or attached onto a detachable/releasable surface and not onto a glass microscope slide. The control cell pellet samples of the present disclosure can be peeled off of the detachable support material and transferred onto non-detachable surface (such as a glass microscope slide) for analysis. These control cell pellet sections are described as "Peel-and-Stick." With this disclosure, it is now possible to stick a control cell pellet section onto any microscope slide containing a test sample such that each test sample contains its own internal positive control. Since each test sample will now contain its own internal positive control there are a number of analyses, corrections, comparisons, and calibrations that can be applied to each sample. It is also possible for the disclosed control cell pellet samples to be prepared by a commercial source and end users efficiently apply such sample to each test ample.

Accordingly, disclosed herein are control cell pellets and their methods of use. For example, universal positive control cell pellets, antigen-retrieval control cell pellets and endogenous peroxidase control cell pellets are described. Uses are also disclosed for these three control cell pellets in immunohistochemistry procedures. Also provided are methods of preparing and using the disclosed quality control device and as well kits including one or more of such devices. Also disclosed are calibrator cell pellets and methods of use as a standard for quantifying the intensity of immunohistochemical staining, for instance for normalizing immunohistochemical staining to a calibrated standard, thereby allowing more accurate quantitation of staining intensity. A calibrator cell pellet can contain a known quantity of a universal positive control antigen, and therefore produces an

expected staining intensity. Thus, it is useful for correcting for both inter- and intra-run variability in IHC assays. Other embodiments are kits for performing the assay.

III. Control Cell Pellets

This disclosure describes methods for making and using control cell pellets for immunohistochemistry assays. The control cell pellets described herein include the universal positive control cell pellet, the antigen-retrieval control cell pellet and the endogenous peroxidase control cell pellet. These three control cell pellets can be used individually or they can be used together, in any combination, as part of an array for simultaneous use in an IHC assay.

IHC assays typically include one or more controls to ensure that each step of the method was performed correctly and the reagents are functioning properly. A standard assay includes a negative control and a positive control. A negative control is usually a reagent that does not contain the specific primary antibody. The negative control reagent is usually placed on a second microscope slide which contains a second portion of the test sample. Since the negative control does not contain the antibody, there is no colored end-product. If a colored end-product is observed then the test is considered invalid. A positive control is usually a tissue that is known by previous testing to contain the antigen of interest. The positive control always produces a positive colored end-product. If the positive control stains negative, then the test is considered invalid.

When the positive or negative control fails to give the expected results, this is an indication that the IHC test is invalid. However, this alone does not provide information as to which part of the IHC staining process was invalid. Since the IHC staining process involves several steps it can be important to determine which specific step specifically was defective in order to correct the error. The control cell pellets described herein can be used to pinpoint the exact cause of the IHC failure.

Cell pellets are formed from cell lines and differ from tissues in several respects. Specifically, cell lines (i) are grown *in vitro*; (ii) are uniform with respect to their biological properties and antigen expression; (iii) provide a limitless supply of control materials; and (iv) are well characterized in terms of their characteristics,

including quantitation of antigen levels. Because of these properties, control cell pellets offer advantages over other types of controls for IHC assays.

A. Universal Positive Control Cell Pellets

Described herein are universal positive control cell pellets that can be used in virtually any IHC procedure regardless of the specific antigen being investigated. The universal positive control cell pellet contains a generic antigen that is not structurally related to the antigen of interest, but nevertheless always stains positive when the IHC procedure has been performed correctly. This provides the same guarantee that the test was valid as does an antigen-specific positive control cell pellet (a cell pellet including cells known to express the antigen of interest).

One of the major limitations of the currently available antigen-specific positive control cells is that they cannot be used universally for all IHC procedures. Currently, only a small number of antigen-specific positive control cells are currently available. However, in IHC there are hundreds of different antigens that are routinely tested for, and for most of these antigens, an antigen-specific positive control cell pellet is not commercially available. Thus, provided herein are universal positive control cell pellets and uses thereof.

B. Antigen-retrieval Control Cell Pellets

Antigen retrieval is a process involving heating a solid support, such as a microscope slide, containing a test sample to an elevated temperature (*e.g.*, about 100°C) for a period of time sufficient to allow chemical modification of the structure of the antigens present in the test sample. This modification frequently results in an improved antigen-antibody reaction. In many cases, no antigen-antibody reaction will occur at all unless antigen retrieval has been performed. In other cases, the antigen-antibody reaction is diminished. Whether the antigen-antibody reaction is improved, unchanged, or diminished is generally dependent upon the specific antigen under investigation. For many antigens, it is known whether the antigen retrieval process is required for antigen-antibody interaction and a successful IHC assay.

In a typical set of IHC assays, some test samples will be exposed to antigen retrieval (if the antigen under investigation so requires) and some tissues will not be

exposed to antigen retrieval. After the slide has undergone the antigen retrieval process, it is not possible to observe any outward visible changes or other indicators that would distinguish this slide from other slides that had not been undergone this process. The only way of distinguishing treated from untreated slides is by accurate labeling and record keeping. However, one common error in IHC occurs by mixing up treated and untreated slides. Another error in IHC occurs when the antigen retrieval procedure is inadequately performed, such as when the desired temperature was not reached, or the desired time interval at elevated temperature was not achieved. It is generally difficult if not impossible to detect such technical errors. Thus, the present disclosure provides an antigen-retrieval control cell pellet that can definitively confirm that antigen-retrieval was performed properly.

C. Endogenous Peroxidase Control Cell Pellets

As part of the IHC staining process, the antigen-positive cells are labeled with a detectable marker (*e.g.*, that can be observed under the microscope). One common method of labeling cells is with the enzyme peroxidase. After the cells have been labeled with peroxidase they are next exposed to a colorless substrate solution, such as diaminobenzidine (DAB). If peroxidase is present, DAB is oxidized to a brown insoluble reaction product that acts to dye the cells. The dyed cells can be readily observed under the microscope. In contrast, if the cells are not labeled with peroxidase, the substrate is not oxidized, and the cells remain unstained.

However, some mammalian cells (including some human cells) contain their own peroxidase enzyme, termed endogenous peroxidase. This endogenous peroxidase gives a false positive stain because the endogenous peroxidase is capable of oxidizing the substrate to form the brown reaction product. Therefore, in some cases it is difficult if not impossible to discern which brown stain was from the peroxidase-labeled cells and which was from the endogenous peroxidase. However, the IHC process can include a step which blocks endogenous peroxidase. Before application of the primary antibody, endogenous peroxidase is blocked by incubation of the test sample in a peroxidase blocking solution, such as 3% hydrogen peroxide. This causes any endogenous peroxidase to be destroyed. At the end of the IHC

staining procedure, any brown staining observed comes from the peroxidase-labeled cells, since there was no endogenous source of peroxidase.

One common mistake in the IHC procedure is to forget to perform the endogenous peroxidase block, or to perform a procedure that is inadequate to block endogenous peroxidase. There are no outwardly visible changes that can identify a slide that has been blocked for endogenous peroxidase. This can only be done by proper labeling and record keeping. Thus, the present disclosure provides an endogenous peroxidase control cell pellet that can definitively identify the status of a slide with respect to its endogenous peroxidase blocking. The cell pellet stains positive when peroxidase blocking was inadequate and stains negative when peroxidase blocking was successfully applied.

Instead of peroxidase, some IHC assays use the enzyme alkaline phosphatase, which is also present in mammalian cells as an endogenous enzyme. IHC assays using alkaline phosphatase generally include a blocking step to eliminate this endogenous activity. Thus, control cell pellets can also be made using cells that express endogenous alkaline phosphatase.

D. Preparation and Use of Control Cell Pellets

Provided herein are methods of preparing a universal positive control cell pellet for an immunohistochemistry assay. In particular examples, the method includes selecting a universal positive control cell line, wherein cells of the cell line express a known antigen; expanding the cells *in vitro*; and collecting and concentrating the cells into a pellet. The conditions and procedures used for expanding cells *in vitro* will vary depending on the cell line selected as the universal positive control; however, cell culture methods are well known in the art. Methods of collecting the cells and concentrating the cells into a pellet, such as by centrifugation, also are well known in the art.

In one embodiment, the antigen is an endogenous antigen. In one example, the endogenous antigen is mouse immunoglobulin. Cell lines expressing mouse immunoglobulin include, but are not limited to, hybridoma cell lines, lymphoma cell lines and plasmacytoma cell lines. Numerous cell lines expressing mouse

immunoglobulin are known and are publicly available, such as from the American Type Culture Collection (Manassas, VA).

Also provided are methods of preparing a universal positive control cell pellet for an IHC assay. In particular examples, the method includes labeling cells *in vitro* with an exogenous antigen and collecting and concentrating the cells into a pellet. In one embodiment, the exogenous antigen is coupled to the cells. Means of coupling the antigen to the cells include, but are not limited to chemical conjugation and physical means. Conjugation techniques are well known in the art (see, for example, Hermanson, G.T., "Bioconjugate Techniques," Academic Press, San Diego, CA, 1996). The exogenous antigen can be any antigen for which a specific antibody exists or can be generated. In one embodiment, the exogenous antigen is a protein. In another embodiment, the exogenous antigen is a hapten. Haptens suitable for use in the methods disclosed herein include, but are not limited to fluorescein, biotin, dinitro phenol and digoxigenin.

The disclosed methods for preparing a universal positive control cell pellet can further include one or more of the following steps: fixing the cell pellet; embedding the cell pellet into a polymeric substance; cutting a section of the embedded cell pellet; and placing the section on a solid support. Methods for fixing and embedding cells, as well as cutting sections of the embedded cell pellets, are described herein and are well known in the art. As described herein, the cell pellets are embedded in a polymeric substance. Any polymeric substance can be used as long as it allows for preparing thin sections of the embedded cell pellets (*e.g.*, the material provides enough rigidity to allow the sample to be sectioned). In one embodiment, the polymeric substance is paraffin. In another embodiment, the polymeric substance is agarose. In another embodiment, the polymeric substance is gelatin.

In other embodiments, the cells can be suspended in a liquid media, such as hot agar, and dispensed onto a detachable surface by a dispensing device, such as a pipette. For example, when the hot agar contacts the cool detachable surface the hot agar solidifies, thereby attaching the cells to the detachable surface.

The solid support can be any solid surface suitable for processing IHC samples. In one embodiment, the solid support is a glass slide. In some embodiments, the solid support further includes a test sample, an antigen retrieval control cell pellet section, an endogenous peroxidase control cell pellet, or a combination thereof.

In one embodiment, the control cell pellets are embedded in and cut from the same polymeric block before placing on the solid support.

Further provided herein are methods for performing an IHC assay. In particular examples, the methods can include fixing a universal positive control cell pellet and test sample, wherein the test sample is a tissue or cell pellet; embedding the universal positive control cell pellet and test sample into a polymeric substance; cutting a section from the embedded universal positive control cell pellet and the embedded test sample; and placing the sections on the same solid support. In one embodiment, the method further includes preparing a universal positive control cell pellet, including selecting a universal positive control cell line, wherein the cells of the cell line express a known antigen; expanding the cells *in vitro*; and collecting and concentrating the cells into a pellet. In another embodiment, the method further includes preparing a universal positive control cell pellet, including labeling cells *in vitro* with an exogenous antigen; and collecting and concentrating the cells into a pellet.

In some embodiments, the solid support further includes an antigen retrieval control cell pellet section, an endogenous peroxidase control cell pellet, or both. In one embodiment, the control cell pellets are embedded in the same polymeric block. In one embodiment, the antigen is an endogenous antigen. In one example, the endogenous antigen is mouse immunoglobulin. In another embodiment, the antigen is an exogenous antigen. The exogenous antigen can be coupled to the cells, such as by chemical or physical means. In some embodiments, the exogenous antigen is a hapten.

IV. Quality Control Device and Uses Thereof

A. Quality Control Device

Disclosed herein are quality control devices that can be used, for example, in an IHC assay. The devices provide a quality control sample, such as a positive control cell pellet section, on a membrane so that the quality control sample can be removed from such surface and transferred onto a second surface (*e.g.*, a glass microscope slide) containing a test sample. Thus, the disclosed device allows each test sample to contain its own internal positive control (*e.g.*, a quality control sample positioned and processed simultaneously on the same slide as the test tissue).

In some examples, the disclosed quality control device includes a quality control sample and a membrane. In one example, the quality control sample includes control cells embedded in a polymeric substance. Any polymeric substance can be used as long as allows for preparing thin sections (*e.g.*, about 2 to 8 microns) of the embedded samples. In one example, the polymeric substance is paraffin. In another example, the polymeric substance is agarose. In other examples, the polymeric substance is gelatin.

In certain examples, the quality control sample is a tissue or cell pellet section, such as one of the disclosed control cell pellet sections. In one example, the quality control sample is a universal positive control cell pellet sample. For example, a universal positive control cell pellet section can be formed of cells expressing an endogenous antigen, such as an immunoglobulin. In other examples, the quality control sample is a universal positive control cell pellet section formed of cells labeled with an exogenous antigen, such as fluorescein, biotin, digoxigenin, or dinitro phenol. For example, the exogenous antigen can be coupled to the cells such as by chemical or physical means. In one example, the exogenous antigen is chemically conjugated to the cells. In additional examples, the quality control sample is an antigen retrieval control cell pellet section. In other examples, the quality control sample is an endogenous peroxidase control cell pellet sample.

The size and the shape of the quality control sample can vary, for example the quality control sample can be circular, square, oval, rectangular or irregular in shape. In some examples, the sample is of a size that allows the quality control

sample to be processed on a standard microscope slide of approximately 1 inch x 3 inches. Thus, in such examples the quality control sample is smaller than 1 inch x 3 inches, such as 0.1 inches to 0.9 inches x 0.1 inches to 2 inches. In other examples, the sample size permits the control sample be placed adjacent a test sample on the same solid support (*e.g.*, a microscope slide), such as size of approximately 1 to 2 millimeters in diameter. In additional examples, the quality control sample is a microtome section of approximately 2 or more microns thick, such as at least 4, at least 6 or at least 8 microns.

In certain examples, the disclosed quality control device includes a membrane. The membrane can be any surface that allows a sample, such as a quality control sample, to be immobilized and subsequently removed without significantly damaging the cellular components of the sample (*e.g.*, causing the sample to wrinkle, tear and the like). In some examples, a membrane includes a first surface that is a detachable surface. A detachable surface is any surface from which a sample can be detached from without significantly damaging the cellular components. Examples of a detachable surface include regenerated cellulose (*e.g.*, cellophane), cellulose acetate, nylon, polycarbonate, paraffin, wax, and fluoropolymer coatings, such as polytetrafluoroethylene and perfluoroalkoxy. In one example, the detachable surface is regenerated cellulose. In another example, the detachable surface is paraffin. In other examples, the detachable surface is a surface coated with a fluropolymer. In certain examples, the membrane is a membrane "strip" which includes multiple quality control samples, such as multiple control cell pellet sections. In certain examples, the membrane strip includes perforations assisting with the detachment of individual quality control devices. It is however readily apparent that other shapes or forms of the membrane can be used with the quality control device, and these alternative forms are also encompassed by the present disclosure.

In additional examples, the quality control device includes a dispenser that is operationally coupled to the membrane for allowing the membrane with the quality control sample to be efficiently dispensed, for example by a user. The size and shape of the dispenser can vary so long as the dispenser is capable of supporting a

quality control device and has a mechanism to detach or dispense the device. In some examples, a dispenser is configured to store multiple quality control devices and allow individual units of such devices to be efficiently dispensed. For example, a dispenser can be designed so that individual quality control devices can be peeled from a membrane strip, such as a membrane strip that had been rolled, in a manner similar to removing a postage stamp from a long row (*e.g.*, roll) of stamps. In other examples, a dispenser includes a cutting mechanism (*e.g.*, a plurality of teeth) on one end of the device allowing individual quality control devices to be separated from the remaining roll of quality control devices. Additional examples of a dispenser can include a dispenser that supports multiple individual strips of membranes each including a quality control sample, so that individual quality control devices can be dispensed without use of a serrated edge.

In some examples, the second surface to which the quality control sample is adhered is a non-detachable surface for permanently adhering the quality control sample. For example, the second surface can be a solid support surface (*e.g.*, a surface sufficiently rigid to provide support to a sample). As previously described, the solid support can be any solid surface suitable for processing IHC samples. For example, a solid support is a rigid, flat, optically opaque and substantially non-fluorescent surface, such as a glass microscope slide or multi-well plates. In additional examples, the solid support fits entirely onto the stage of a microscope and accommodates at least one quality control sample and test sample to allow simultaneous processing. For example, the solid support can include a test sample, a universal positive control cell pellet sample, an antigen retrieval control cell pellet sample, an endogenous peroxidase control cell pellet sample or combinations thereof.

B. Methods of Preparing and Using the Quality Control Device

A feature of this disclosure is to prepare a quality control sample, such as a cell pellet section, in a way that it can first be attached to a surface in a temporary manner, and later removed (*e.g.*, peeled off) from this temporary surface and attached to another permanent surface. The rationale for this approach has been more fully explained above. Thus, a goal of the present disclosure is to provide a

device that allows each test sample to contain its own internal positive control (*e.g.*, a quality control sample positioned and processed simultaneously on the same slide as the test sample).

Accordingly, methods of preparing and using a quality control device are also described herein. For example, methods for preparing a quality control device can include immobilizing a quality control sample on a first surface, in which the quality control sample includes a cell pellet section and the first surface is a detachable surface. In some examples, the methods also involve preparing the quality control sample by selecting a quality control sample cell line, wherein the cells of the cell line express a known antigen; expanding the cells *in vitro*; and collecting and concentrating the cells into a cell pellet. In additional examples, the methods include removing the quality control sample from the first surface. The methods can also include adhering the quality control sample to a second surface, such as a glass microscope slide. A detailed description for such methods is described elsewhere herein.

V. Calibration

Disclosed herein is a calibrator cell pellet (also referred to as calibrator cells) that is useful for calibrating IHC staining intensity. A deficiency of IHC is its lack of standards, making accurate quantitation difficult, if not impossible. The staining results achieved by IHC are typically viewed by a trained observer under the microscope, and the staining intensity is subjectively scored usually on a scale of 0 to 4, with 0 being negative and 4 being intensely positive. Because there are no standards for comparison, prior to this disclosure, the method was not quantitative, and there were no methods to correct for intra- and inter-run variability.

Because of this variability, a number of potential errors can be introduced into the staining procedure. If these errors are undetected, they may lead to erroneous results. Usually, such errors are technical errors that arise when two or more slides are unintentionally treated differently during the staining protocol. The most common source of error is inconsistency in the incubation time following application of one or more IHC reagents to different slides. Even small inconsistencies in incubation time can result in one of the slides staining slightly

more strongly or slightly more weakly than another slide. This type of error is known as intra-run variability (*e.g.,* the difference in staining between slides that were all stained at the same time under more or less similar conditions.) Another source of error occurs when one test tissue is compared to another test tissue. Sometimes it is necessary for a laboratory to test multiple patient tissue samples simultaneously. However, because of the known intra-run variability among samples such comparisons are not always accurate.

Attempts to minimize variability in IHC staining require stringent adherence to the IHC protocol, the proper use of controls, and automation. Even with these precautions, a degree of variability inevitably occurs and is accepted as inherent to the method. Inter-run variability is the variability between slides that were stained at different times and possibly under different conditions. Inter-run variability is usually greater than intra-run variability.

One or more of these deficiencies in the IHC procedure can be corrected using the calibrator cell pellets described herein. Calibrator cells provide a fixed standard to which the IHC staining results are compared. IHC staining results are then normalized to the calibrated standards such that intra- and inter-run variability are significantly reduced, or even eliminated.

A calibrator cell pellet standard is similar to the positive control described above in that it contains the molecule of interest. However, a calibrator cell pellet standard also has a known amount of the molecule (*e.g.,* antigen) of interest. Frequently, a quantitative assay will contain more than one standard with each standard having a different (but known) amount of the molecule of interest. When the staining is completed, each of the standards will exhibit a color which is proportional to the amount of the molecule contained in that standard. By first measuring the amount of color (such as in a spectrophotometer, for example) a graph can be made by plotting a standard curve of the optical density (color) of the standard on the Y-axis and the molecule concentration of the X-axis. Next, the optical density of the sample being tested is found on the graph and the corresponding molecule concentration is read. This method provides an objective and quantitative method of determining the concentration of an unknown molecule.

Calibrator cells provide essentially the same function for IHC that standards provide for quantitative diagnostic tests. The calibrator cells provide an expected staining result, and thus have an expected value. Generally, a calibrator cell pellet is placed on each microscope slide with the test tissue to be stained, and thus becomes physically linked to the test specimen. The slide is then stained by the IHC method, resulting in the simultaneous staining of both the calibrator cells and the test specimen. The staining is then measured in the calibrator cells, and this measurement is called the observed value. Based on the inherent variability of the IHC method, the slides may sometimes stain a little darker than expected, while at other times the slides may stain a little lighter than expected. This inherent variability is also present in the calibrator cells, and affects their staining, as well. However, the expected staining intensity of the calibrator cells is known, so if a divergence from the expected is observed, it can be inferred that this divergence represents variability which is not only present in the calibrator cells but in the test specimen as well. This divergence is the difference between the expected value and the observed value.

Not only do the calibrator cells detect variability, but they will also allow for correction of the variability. Image analysis permits the collection of digital images of the calibrator cells and test specimen, and computer algorithms are used to measure the amount of staining. The amount of staining present in the calibrator cells is measured (observed value) and compared to the known expected value. If variability is detected, then the divergence (either positive or negative) from the expected value is calculated. This calculated divergence then becomes a correction factor that is applied to the digital image of the test specimen to remove the variability. This is called normalizing the results to a calibrated standard.

Once all of the digital images have been normalized to the calibrated cells, the images can be accurately quantified for staining intensity, and the staining results from different test specimens can be compared to each other.

VI. Kits for IHC

Kits for performing IHC are also provided. In some examples, the kit includes one or more of the disclosed quality control devices. For example, a kit can

include at least one of the quality control devices disclosed herein and instructions for using the device. In certain examples, a kit includes at least one quality control device including one or more quality control samples (such as one or more universal positive control cell pellets, antigen-retrieval control cell pellets, endogenous peroxidase control cell pellets, calibrator cell pellets or a combination thereof). In some examples, the kit also includes reagents for performing IHC (*e.g.*, buffers, detection agents, and the like, which can be in separate containers) and at least one solid support, such as a glass microscope slide, to which the test sample and one or more quality control samples can be attached.

EXAMPLES

Example 1

Immunohistochemistry Method

This example describes a general procedure for performing an IHC assay to detect a molecule of interest in a test sample. Typically, the IHC assay includes the test sample and one or more control samples, such as a universal positive control, antigen retrieval control, endogenous peroxidase control, or combination thereof as described herein.

The IHC staining procedure generally includes five basic steps. First, a primary antibody specific for the molecule of interest (*e.g.*, target antigen) is applied to a solid support containing test and control samples, such as a microscope slide, such that the antibody overlays the control and test samples. The antibody is allowed to incubate with the samples for a sufficient length of time to allow the antigen-antibody reaction to occur. For the purposes of this example, the primary antibody can be any known antibody specific for the antigen of interest (*e.g.*, a mouse monoclonal antibody). The slide is rinsed to remove any unbound primary antibody. A secondary antibody, such as an anti-mouse immunoglobulin antibody, is applied to the solid support such that the secondary antibody overlays each of the control and test samples. If the primary antibody has bound to the cell pellet or tissue sample *via* an antigen-antibody reaction, then the secondary antibody will now bind to the primary antibody via a second antigen-antibody reaction. If primary antibody has not bound to the sample, then the secondary antibody will likewise not

bind. The secondary antibody is linked to an enzyme, dye, or other detectable label. The secondary antibody can be directly linked to a detectable label, or it can be indirectly linked through a series of steps. In either case, the ultimate result is a physical link between the detectable marker and the secondary antibody. Thus, only if all the steps have been performed correctly will the detectable marker be present. The final step involves detection of the marker. In IHC, a detectable marker is frequently an enzyme such as peroxidase, which then reacts with a colorless substrate converting it into a colored end-product. The presence of the colored end-product indicates that the molecule of interest is present in the test sample.

Example 2

Universal Positive Control Cell Pellet Sample Preparation and Uses

This example describes the preparation of a universal positive control cell pellet sample. A mouse lymphoma cell line expressing mouse IgG_{2A} (obtained from the American Type Culture Collection (ATCC; TIB-12)) was grown *in vitro* by placing the cells in a flask containing a nutrient medium composed of RPMI plus 10% bovine calf serum, nonessential amino acids, and L-glutamine. The cells were incubated for three days to allow cell replication. After the desired number of cells was achieved, the cells were harvested, and concentrated into a cell pellet by centrifugation at 100 x *g* for 10 minutes. The cell pellet was fixed in 95% ethanol for 10 minutes to stop all metabolic activity and to immobilize the antigens within the cells. The fixed cell pellet was then dehydrated in 100% ethanol and cleared in xylene. The cell pellet was dispersed into 200 µl of molten paraffin at 57°C and drawn into the bore of a warmed (57°C) glass Pasteur pipette. The pipette contents were then quickly discharged into a recipient paraffin block that had been prepared to accept the cell sample. The recipient paraffin block was prepared by removing a cylindrical core of solid paraffin from the block leaving an empty column measuring approximately 2 mm in diameter. The contents of the Pasteur pipette containing the molten paraffin and cell sample was quickly pipetted into the empty column and solidified at room temperature. The paraffin block, with the embedded cell pellet, was cut into thin slices of approximately 4 microns in thickness. The thin slices

were first floated onto a water bath to straighten and were then collected onto a cellophane medium and laid out flat on top of the cellophane medium. The cell pellet section was temporarily attached to the cellophane medium by drying the section, followed by exposing the section to an increased temperature (56°C for five minutes), such that the paraffin melted onto the slide affixing the cell pellet section contained in the paraffin to the cellophane medium. The cell pellet section was then cooled by exposing the section to room temperature (approximately 22°C) such that the paraffin re-solidified. The section was stored at room temperature. This procedure allows a quality control sample to be temporarily attached and subsequently removed from a first surface to adhere to a second surface without damaging the quality control sample.

A test sample, such as a test tissue or test cell pellet, is prepared in a similar manner as described for the universal positive control cell pellet. The paraffin slices of the universal positive control cell pellet and the test sample are affixed to the same slide and processed using the immunohistochemistry method described above in Example 1. The universal positive control cell pellet expressing mouse immunoglobulin will stain positive any time the IHC procedure has been performed correctly. These positive control cells will stain positive regardless of the staining reaction of the test tissue. By virtue of the fact that the control cells contain mouse immunoglobulin, they will stain positive whenever the secondary antibody of the IHC procedure is applied. As described above, the secondary antibody is an anti-mouse immunoglobulin antibody which will always react with the mouse immunoglobulin present on the control cells. A positive staining reaction of the control cells demonstrates that (i) the sample was treated with the secondary antibody and all subsequent steps including any tertiary reactions, enzyme, and substrate were performed correctly; (ii) the protocol from the secondary antibody onward was appropriately followed; and (iii) all reagents from the secondary antibody onward were functional. A positive reaction does not guarantee that the first step (the primary antibody) was performed correctly. Nevertheless, about 80% of the procedure is controlled for by using the universal positive control cell pellet.

Example 3

Universal Positive Control with an Exogenous Antigen

This example provides an exemplary procedure for preparing a universal positive control cell pellet using the exogenous antigen fluorescein. The cells are prepared similarly to the preparation described in Example 2, with the exception that after the cells are harvested from the tissue culture flasks, they are labeled with fluorescein (Sigma-Aldrich, St. Louis, MO). In this example, the universal positive control cell pellet is prepared by chemical conjugation of the fluorescein molecule to the surface of the control cells according to the method of Butcher *et al.* (*J. Immunol. Methods* 37(2):109-21, 1980). Viable cells are incubated with free fluorescein at a concentration of approximately 10 μg/ml. The fluorescein is added to the cell growth medium 24 hours prior to harvesting the cells. After harvesting, the cells are separated from unbound fluorescein by centrifugation for 10 minutes at 100 x *g*. The universal positive control cells and the test tissue can then be processed into paraffin and adhered to a cellophane membrane in a manner similar to the method described in Example 2.

In this example, the primary antibody reagent (the reagent containing the antibody to the molecule of interest) includes a second antibody to fluorescein. The second antibody does not interfere with the primary antibody to the antigen of interest. The purpose of the second antibody is to react with the universal positive control cells. When the IHC staining procedure is followed as described above, the test tissue may or may not stain positively, depending on the presence or absence of the antigen of interest, but the universal positive control cells will stain positive by virtue of the reaction of the anti-fluorescein antibody with the fluorescein molecule on the surface of the control cells. Thus, a positive stain on the universal positive control cell pellets indicates that (i) a primary antibody was applied; (ii) all steps subsequent to the primary antibody were successfully completed; (iii) all reagents were functional; and (iv) the correct protocol was followed.

Example 4

Antigen-Retrieval Control Cell Pellets

This example describes the use of a cell line that expresses mouse immunoglobulin as an antigen-retrieval control for an immunohistochemistry assay. The cell pellet is prepared as described above (Example 2) for the universal positive control cell pellet. Briefly, a cell line expressing mouse immunoglobulin, such as a hybridoma cell line, plasmacytoma cell line or a lymphoblastoma cell line, is expanded *in vitro* to generate the desired number of cells. After expansion, the cells are collected and pelleted.

The cell pellet is chemically fixed as part of the preparation process in order to stop all metabolic activity and to preserve the chemical and structural integrity of the cells. If the fixative is 10% formalin, the expressed mouse immunoglobulin will be chemically modified, resulting cross-linking of the immunoglobulin molecules. Cross-linking of mouse immunoglobulin renders the antigens undetectable by standard IHC staining methods. However, if antigen-retrieval is performed on the chemically modified cells prior to the IHC staining procedure, the mouse immunoglobulin antigens are restored to the native state and can now be stained by the IHC staining procedure.

Thus, positive staining of the antigen retrieval control cell pellet indicates antigen retrieval was performed correctly. The absence of staining indicates the antigen retrieval process did not occur, or was performed incorrectly.

Although in most cases no staining of the antigen retrieval control means that antigen retrieval was not performed, it could also result from a technical error. Therefore, the antigen-retrieval control must be interpreted in the context of the universal positive control. The universal positive control must be positive in all situations before the antigen-retrieval control can be considered valid. Table I provides a summary of the interpretation of the controls.

Table I

Interpretation of Control Cell Pellet Staining

Antigen-retrieval positive control	Universal positive control	Interpretation
+	+	Antigen retrieval performed IHC test valid
-	+	Antigen retrieval not performed IHC test valid
+	-	Antigen retrieval irrelevant IHC test invalid
-	-	Antigen retrieval irrelevant IHC test invalid

Example 5

Endogenous Peroxidase Control Cell Pellets

This example describes the preparation and use of endogenous peroxidase control cell pellets. The endogenous peroxidase control cell pellets utilize cells that contain endogenous peroxidase (or pseudo-peroxidase) activity. In this example, cells may be chosen from any of a number of suitable cell lines that contain such activity.

One type of cell containing endogenous peroxidase is a red blood cell. Because red blood cells can be obtained from blood in large quantities, it is possible to harvest red blood cells from a donor animal rather than grow the cells *in vitro*. However, not all red blood cells are appropriate sources for preparing cell line controls. Mammalian red blood cells do not contain a nucleus, and are therefore, too fragile to embed into paraffin according to the methods described above. In contrast, avian red blood cells are nucleated and are structurally strong enough to be embedded into paraffin.

In this example, chicken red blood cells are used for the endogenous peroxidase control cell pellet. Chicken red blood cells are readily available through commercial sources.

The chicken red blood cells are prepared, embedded into a paraffin block, cut and placed on a microscope slide, as described above in Example 1. During the IHC staining process the slides are exposed to an endogenous peroxidase blocking step. If this procedure is adequate, the endogenous peroxidase control will stain negative.

If this procedure is inadequate, the endogenous peroxidase control will stain positive.

Example 6

Control Cell Pellet Array

This example describes an immunohistochemistry assay in which a test sample, universal positive control cell pellet, antigen-retrieval control cell pellet, endogenous peroxidase control cell pellet, or a combination thereof, are attached to the same solid support (such as a glass slide) for processing (see FIG. 4). This example further describes embedding two or more of the control cell pellets into a single paraffin block. This is termed a cell pellet array. The cell pellet array permits all three controls to be placed on the same slide as the test sample and stained at the same time as the test sample. Table II provides an interpretation of staining results that can be obtained from the control cell pellets.

Table II

Interpretation of Control Cell Pellet Array Staining

Universal positive control	Antigen-retrieval positive control	Endogenous peroxidase positive control	Interpretation
+	+	-	Antigen retrieval performed IHC stain valid Peroxidase blocking valid
+	-	-	Antigen retrieval not performed IHC stain valid Peroxidase blocking valid
+	+ or -	+	IHC stain valid Peroxidase blocking invalid
-	+ or -	+ or -	IHC stain invalid

The method for preparing a control cell pellet array includes the following steps. Control cell pellets are prepared using standard histological techniques and embedded into a paraffin block as described above. Preferably, the control cell pellets include a universal positive control cell pellet, an antigen-retrieval control cell pellet and an endogenous peroxidase control cell pellet. In this example, the universal positive control cell pellet is prepared from a cell line expressing mouse

immunoglobulin and is fixed in ethanol. The antigen-retrieval control cell pellet is prepared from a cell line expressing mouse immunoglobulin and fixed in formalin. The endogenous peroxidase control cell pellet prepared from chicken red blood cells.

The control cell pellets can be embedded individually into paraffin blocks or all together in the form of an array. The paraffin block containing one or more of the control cell pellets is cut into a thin paraffin section (*e.g.*, 2 to 8 microns), which is attached to a microscope slide. A test tissue sample is prepared and embedded into another paraffin block using standard histological methods. The paraffin block containing the test tissue is cut into a thin paraffin section (*e.g.*, 2 to 8 microns) which is attached to the same microscope slide as the control cell pellets. In this way, both the test tissue and the control cell pellets are attached to the same microscope slide. The microscope slide is then stained by an IHC method for an antigen suspected of being present in the test sample.

The IHC assay is performed essentially as follows. If needed, antigen retrieval is performed on the slide. The slide is then treated with an endogenous peroxidase inhibitor. Next, a first primary antibody (mouse monoclonal antibody) against the antigen of interest is applied to the slide. An anti-mouse immunoglobulin secondary antibody is then applied, which will bind to the primary antibody and to the universal positive control cell pellet by virtue of the fact that these cells express mouse immunoglobulin. The secondary antibody will also bind to the antigen-retrieval positive control cell pellet if antigen retrieval was performed, but will not bind to the antigen-retrieval positive control cell pellet if antigen retrieval was not performed.

In this example, the secondary antibody is linked to a peroxidase enzyme, therefore a peroxidase substrate added to the sample to react with the peroxidase enzyme to produce a colored end-product that stains any cells containing the suspected antigen. The peroxidase substrate will simultaneously stain the endogenous peroxidase control cell pellet if the endogenous peroxidase step was inadvertently omitted, or the treatment was inadequate. However, the endogenous

peroxidase control cell pellets will not stain if the endogenous peroxidase blocking step was adequate.

Next, the staining reaction of the test tissue and control cell pellets is examined under a microscope to evaluate the IHC staining reaction. The slides can be reviewed by conventional observation, or the slides can be analyzed automatically by use of computer-aided image analysis system.

Example 7

Preparation of Calibrator Universal Positive Control Cell Pellet

This Example describes a method of preparing a calibrator universal positive control pellet. In one embodiment, a cell line is grown *in vitro* by placing cells in a flask or other tissue culture chamber containing a nutrient medium and incubating them for several days so the cells replicate in the flask and increase in number. After the desired number of cells is achieved, the cells are harvested and concentrated into a cell pellet. The cell pellet is fixed, and the fixed cell pellet is then embedded into a paraffin block using standard histological methods. The paraffin block, with the enclosed cell pellet, is then cut into thin slices of approximately 4 microns thick. The slice containing the calibrator cells is then placed on one section (*e.g.,* end) of a glass microscope slide, and a second paraffin slice containing a test tissue is mounted on a different section (*e.g.*, the other end) of the glass slide.

Thus, the general steps of preparing the calibrator positive control cell pellet include (1) growing the cells *in vitro,* (2) harvesting the cells and concentrating them into a cell pellet, (3) fixing the cell pellet with a fixative, such as 95% ethanol, (4) dehydrating the cell pellet and embedding the cell pellet into a paraffin block, (5) cutting a thin slice off the paraffin block that includes a portion of the cell pellet, (6) allowing the paraffin slice to spread out by floating it on top of a water bath (7) attaching the floating paraffin section containing the calibrator cells onto a microscope slide, (8) attaching a test tissue specimen onto the same microscope slide (but at a different location), (9) staining both the test tissue and the calibrator cells using IHC or another staining procedure, and (10) analyzing the staining results of

the test tissue and the calibrator cells by either visual observation under a microscope or by image analysis.

Example 8

Use of Positive Control Cell Pellet Samples

This example describes the use of universal positive control samples prepared in the above Examples. In order to attach a universal positive control sample onto another medium, such as a glass slide, forceps were used to remove the cell pellet from the cellophane membrane and place such pellet onto the second medium. For example, a universal positive control sample was peeled off the cellophane membrane with the aid of forceps, placed onto a glass microscope slide containing the test tissue and permanently affixed to a glass slide next to a test sample. The permanent attachment was achieved by placing the cell pellet into a 10 µl droplet of water that had been placed onto the glass slide. The slide was then exposed to an elevated temperature (*e.g.*, 57°C) to evaporate the water and melt the paraffin. This process permanently attached the universal positive control cell pellet sample onto the glass slide.

FIG. 1 shows a microscopic view of a universal positive control cell pellet sample after it has been stained. The staining procedure utilized a mouse monoclonal antibody to vimentin (V9). Slides were first blocked for endogenous peroxidase activity by incubation for five minutes at room temperature with 3% hydrogen peroxide. Slides were then rinsed with phosphate buffered saline and incubated for 10 minutes at room temperature with vimentin antibody (V9). Following incubation with the primary antibody, slides were rinsed in phosphate buffered saline and incubated for 10 minutes at room temperature with the secondary antibody (Envision Plus-HRP, Dako Corp., Carpineria, CA). Slides were then rinsed with phosphate buffered saline, and a substrate of diaminobenzidine (Dako Corp.) was added for five minutes at room temperature. Slides were then counterstained with hematoxylin and mounted with a glass coverslip in preparation for microscopic examination. FIG. 1 illustrates the individual cells that comprise the cell pellet. The brown color around each cell was indicative of a positive immunohistochemical

reaction.

In addition, a universal positive control, which expresses endogenous immunoglobulin, was affixed to a glass microscope slide adjacent to a human tonsil test sample. FIG. 2 provides a microscopic view (10x) of the human tonsil test sample after immunohistological staining for the vimentin antigen. Previous studies have demonstrated that the vimentin antigen is found in various tissue elements contained within tonsil. A positive reaction occurred as indicated by the brown-staining elements. FIG. 3 shows a microscopic view (40x) of the universal positive control cell pellet. These control cells contained naturally occurring mouse immunoglobulins which stain positive whenever the immunohistological staining has been completed successfully. Positive staining was indicated by the brown color reaction. Taken together, these data indicate that the immunohistochemical staining procedure was completed successfully and that the observed vimentin staining does in fact represent specific staining, and not background, artifact, or other error in the immunohistochemical staining method.

Example 9

Calibration of IHC Using a Calibrator Universal Positive Control

This Example provides an exemplary method for calibrating IHC staining using a calibrator cell pellet. Briefly, a microscope slide that has mounted on it a test sample and a calibrator cell pellet (*e.g.,* see Example 7) is stained such that both the calibrator cells and test specimen are simultaneously stained under identical conditions. There are many variations of the IHC method, and any specific method can be used. All IHC methods produce the same end result: a positive stain if the antigen of interest was present in the test specimen and a negative stain if the antigen of interest was not present in the test specimen.

When the calibrator cells stain positive, this is indicative that the IHC method was successful. In this case the calibrator cells act as a positive control. In addition, the calibrator cells contain a known level of antigen that provides an expected level of positive staining. Each time the calibrator cells are stained they, should theoretically provide the same expected level of staining.

In some embodiments, the level of staining is determined using image analysis, which is a process of measuring the amount of staining in a tissue by using a camera (*e.g.,* a CCD camera) to capture an image and then using a computer to analyze the image. Image analysis is more precise in quantifying staining compared to simple visual analysis by the unaided human eye.

Since the theoretical amount of staining of the calibrator cells is known, any deviation from this theoretical limit (such as might occur due to intra-run variability) can be analyzed by image analysis, and a correction factor is calculated. This same correction factor is then applied to the staining intensity measured for the test sample. This process normalizes the test tissue to its internal calibration standard. By normalizing each test sample, the usual intra- and inter-run variability can be eliminated. Not only does this provide more accurate results, but it also allows comparisons of one test sample to another test sample.

In one embodiment, the calibration is carried out manually. The method can include (1) evaluating the staining results of the calibrator cells by visual observation under a microscope, (2) comparing the observed staining results of the calibrator cells to the expected staining results (for instance, the expected staining results may be in the form of a digital image that displays the expected staining pattern), (3) estimating the deviation from the optimal staining pattern by assigning a score of -4 to +4, wherein 0 represents no deviation and +1 to +4 represent overstaining and -1 to-4 represent understaining, (4) evaluating the staining results of the test sample and assigning it an initial value of 0 to 4 based on its staining intensity, with 0 being negative and 4 being strongly positive, and (5) combining the score from step 3 with the score from step 4 to come up with a corrected score. The combined score cannot be less than 0 or greater than 4.

In another example, calibration is by image analysis. The method can include evaluating the staining of the calibrator cells by an image analysis method using an algorithm to quantify the observed staining intensity. A common image analysis algorithm assigns a score of 0 – 255 with 0 being the most intensely stained and 255 being negative. The observed staining of the calibrator cells is then compared to the expected staining, and a correction factor is calculated based on the

deviation between the observed staining of the calibrator cells and the expected staining. The staining of the test specimen is then evaluated by an image analysis algorithm and a value is assigned (usually between 0 – 255). The correction factor is then applied to the test specimen to normalize the results to the calibrator cells and a new stain value is calculated.

In one example (as shown in FIGS. 5-8), test samples that were under- and over-stained were normalized using the calibrator cell pellets. In FIGS. 5-8, serial sections of the same tissue were cut from the same paraffin block and mounted on separate microscope slides. Because all three test sections were cut from the same sample, they would be expected to stain with nearly identical results if the same staining protocol is used with each. Calibrator cells were mounted on each slide along with the test specimens. In order to simulate staining variability, slide 1 was under-incubated with the primary antibody in order to produce an under-stain, slide 2 was incubated in the primary antibody for the correct amount of time, and slide 3 was over-incubated with the primary antibody in order to produce an over-stain. The staining variability depicted in FIGS. 5-8 simulates the variability that might be encountered with the IHC method.

FIG. 5 shows a comparison of the staining intensity of the calibrator cells on each of the three slides. The histogram beneath each panel shows the calculated staining for the calibrator cells. FIG. 6 shows the staining intensity for each test sample on each of the three slides. The three test samples were stained concurrently with their respective calibrator cell pellets. The lower panels show histograms for the staining of each tissue. FIG. 7 shows the corrected IHC results that have been adjusted using the correction factor derived from the calibrator cell pellet staining. All three test samples show similar staining in the lower (corrected) panels. FIG. 8 shows the adjusted histograms for the test samples. The adjusted final scores for each histogram were 171, 175, and 171, respectively. This result demonstrates that the application of the correction factor generated from the calibrator cell pellet data yields essentially identical values from the under-stained, over-stained, and normal test specimens.

In view of the many possible embodiments to which the principles of the disclosure may be applied, it should be recognized that the illustrated examples are only examples of the disclosure and should not be taken as a limitation on the scope of the disclosure. Rather, the scope of the disclosure is defined by the following claims. I therefore claim as my invention all that comes within the scope and spirit of these claims.

I claim:

1. A method for performing an immunohistochemistry assay, comprising:

(i) providing a first universal positive control cell pellet section and a first test sample on same solid support, wherein the first universal positive control cell pellet section contains a known amount of a known antigen;

(ii) performing immunohistochemistry on the first universal positive control cell pellet section and the first test sample;

(iii) analyzing immunohistochemical staining intensity of the first universal positive control cell pellet section and the first test sample;

(iv) assigning a numerical value to the immunohistochemical staining intensity of the first universal positive control cell pellet section;

(v) assigning a numerical value to the immunohistochemical staining intensity of the first test sample;

(vi) comparing the immunohistochemical staining intensity of the first universal positive control cell pellet section to an expected value for the first universal positive control cell pellet section, thereby providing a deviation between the immunohistochemical staining of the first universal positive control cell pellet section and the expected value;

(vii) calculating a correction factor based on the deviation between the immunohistochemical staining of the first universal positive control cell pellet section and the expected value; and

(viii) using the correction factor to normalize the numerical value assigned to the immunohistochemical staining intensity of the first test sample to the expected value for the first universal positive control cell pellet section.

2. The method of claim 1, wherein analyzing the immunohistochemical staining intensity of the first universal positive control cell pellet section and the first test sample comprises visual observation.

3. The method of claim 1, wherein analyzing the immunohistochemical

staining intensity of the first universal positive control cell pellet section and the first test sample comprises digital image analysis.

4. The method of any of claims 1-3, wherein the solid support is a microscope slide.

5. The method of claim 4, further comprising performing the method on a second universal positive control cell pellet section and a second test sample, wherein the first universal positive control cell pellet section and the first test sample are provided on a first slide, and wherein the second universal positive control cell pellet section and the second test sample are provided on a second slide.

6. The method of claim 5, further comprising comparing the normalized value assigned to the immunohistochemical staining intensity of the first test sample to the normalized value assigned to the immunohistochemical staining intensity of the second test sample.

7. The method of claim 5 or 6, wherein each slide comprises no more than one universal positive control cell pellet section.

8. The method of any of claims 1-7, wherein the antigen is an endogenous antigen.

9. The method of any of claims 1-7, wherein the antigen is an exogenous antigen.

10. The method of claim 8, wherein the endogenous antigen is an immunoglobulin.

11. The method of claim 9, wherein the exogenous antigen is fluorescein.

12. The method of claim 9, wherein the exogenous antigen is chemically conjugated to cells of the universal cell pellet control section.

13. The method of claim 4, wherein the slide further comprises at least one of an antigen retrieval control cell pellet sample and an endogenous peroxidase control cell pellet sample.

14. The method of any of claims 1-14, wherein calculating the correction factor based on the deviation between the immunohistochemical staining of the first universal positive control cell pellet section and the expected value comprises calculating a difference between a mean observed staining intensity of the first universal positive control cell pellet section and a mean expected value.

15. The method of any of claims 1-14, wherein normalizing the numerical value assigned to the immunohistochemical staining intensity of the first test sample to the expected value for the first universal positive control cell pellet section comprises multiplying a value assigned to the immunohistochemical staining intensity of the first test sample by the correction factor.

16. A kit for performing the method of any of claims 1-15, wherein the kit comprises a solid support comprising the universal cell pellet control section and instructions for carrying out the method.

17. The kit of claim 16, further comprising at least one of a primary antibody and a secondary antibody.

18. The kit of claim 17, where the primary antibody comprises an anti-universal positive control antibody.

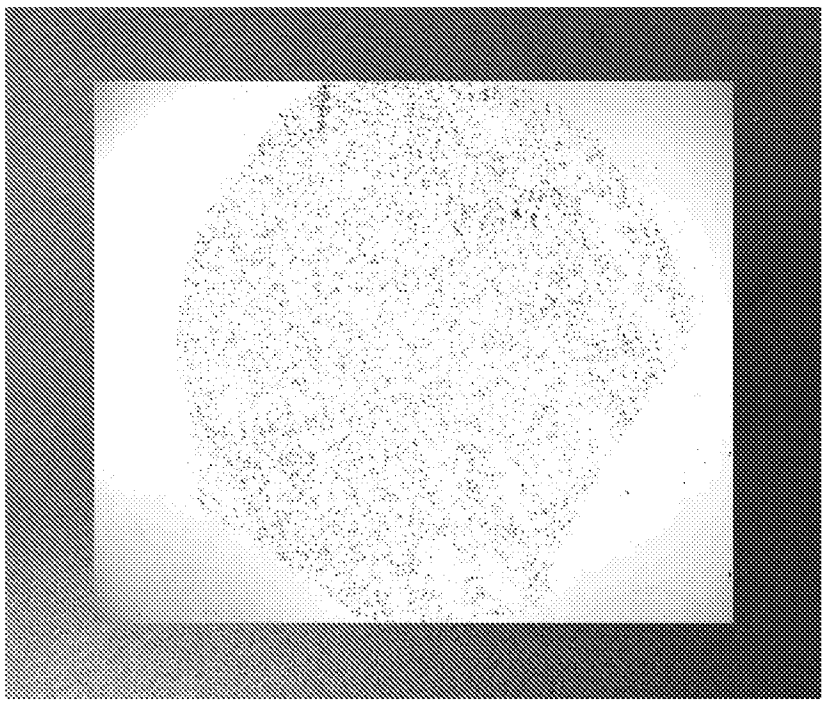

FIG. 1

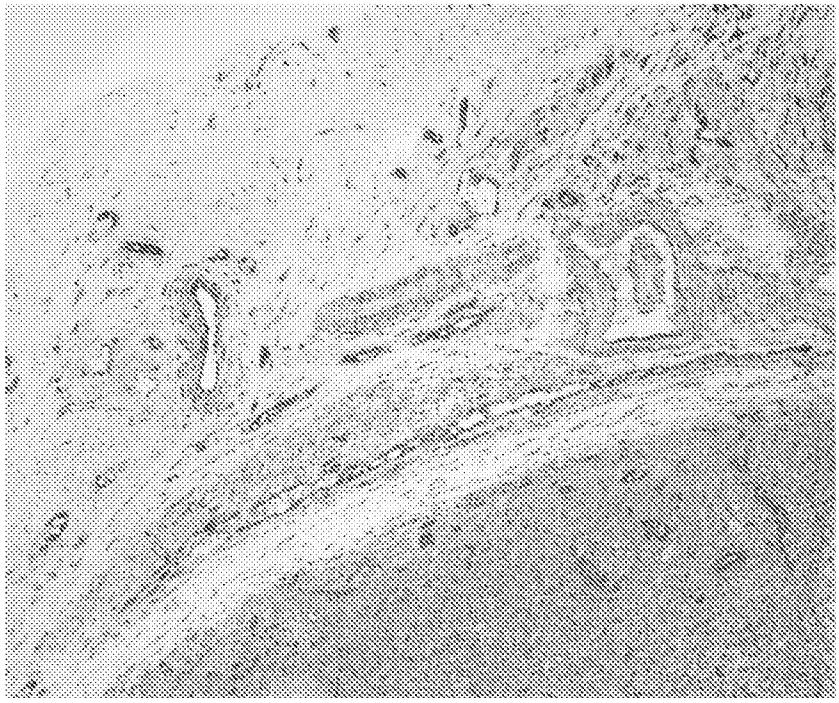

FIG. 2

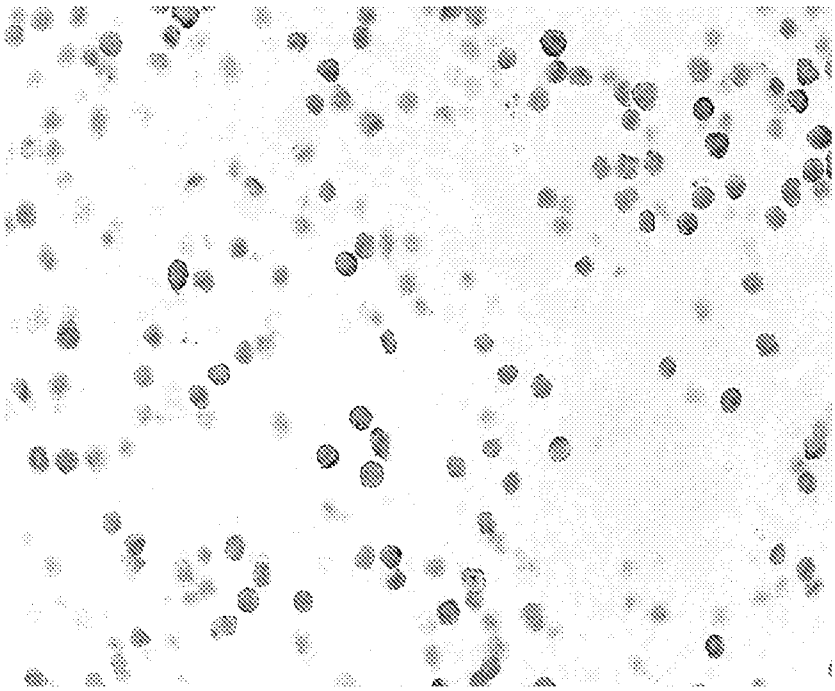

FIG. 3

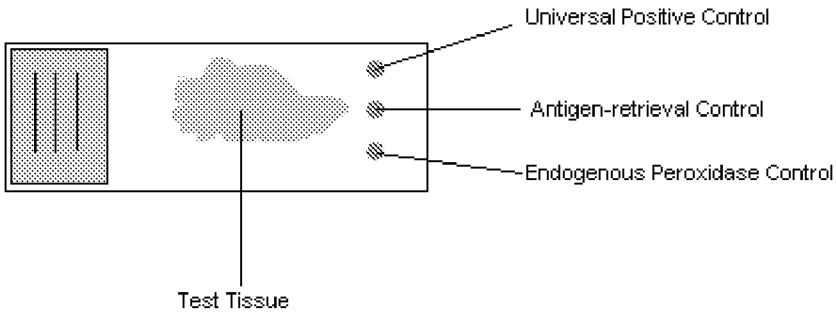
Universal Positive Control
Antigen-retrieval Control
Endogenous Peroxidase Control
Test Tissue

FIG. 4

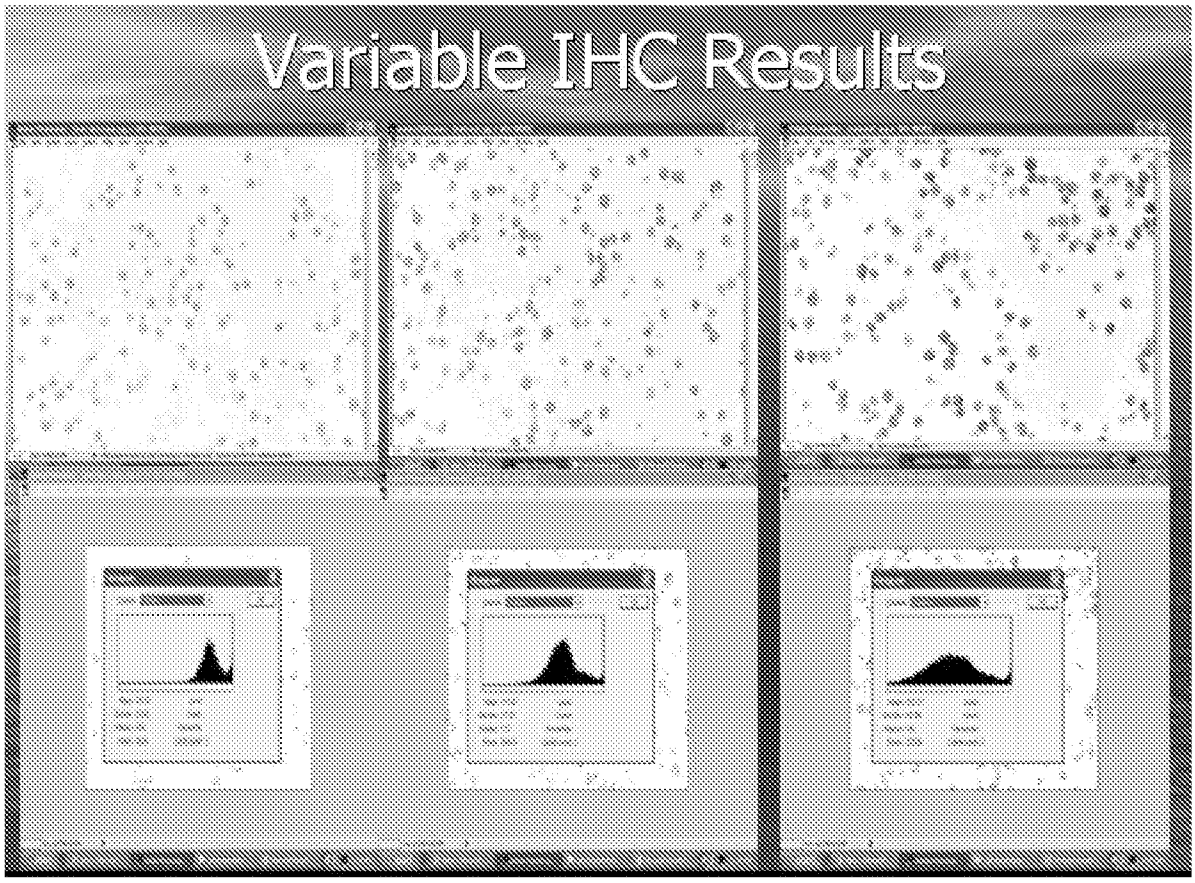

FIG. 5

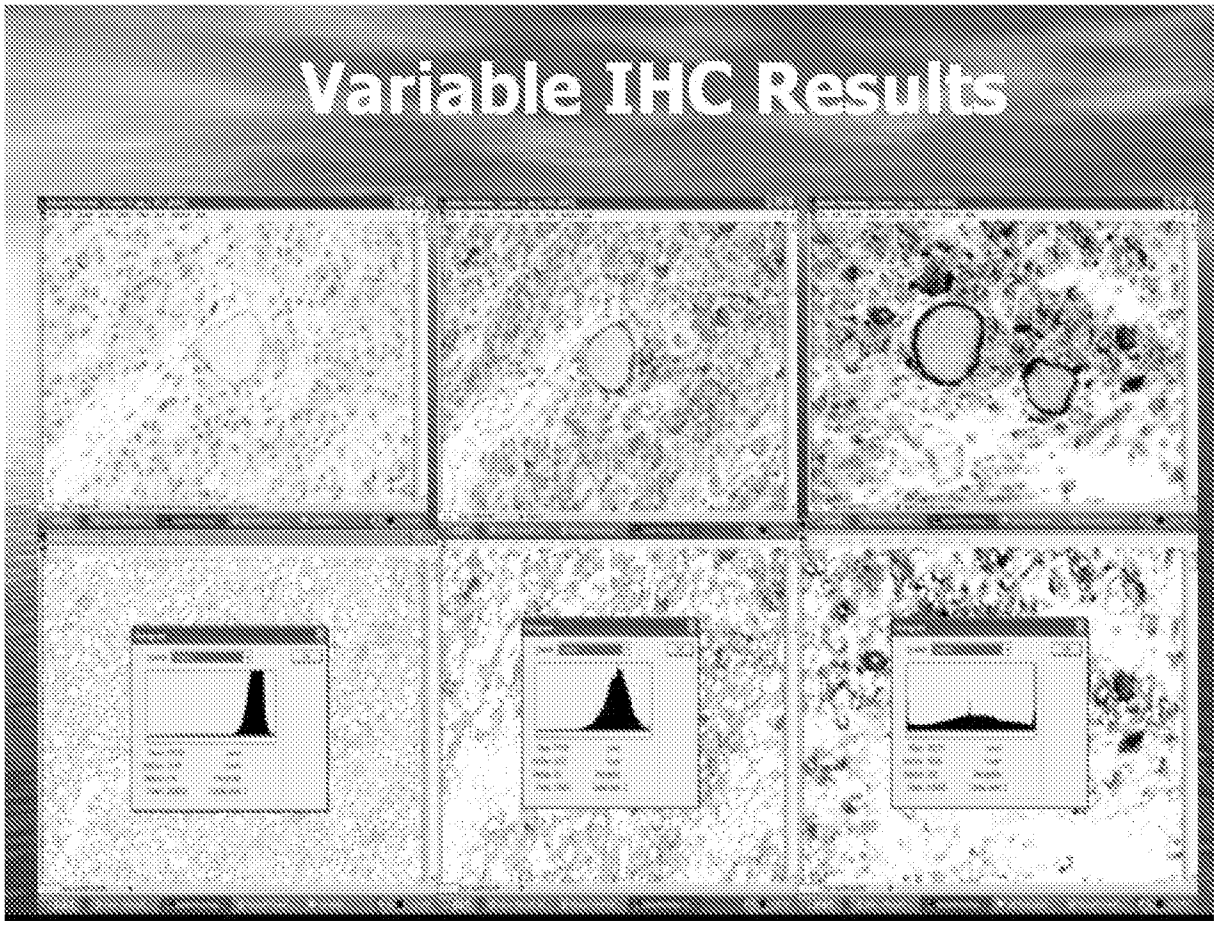

FIG. 6

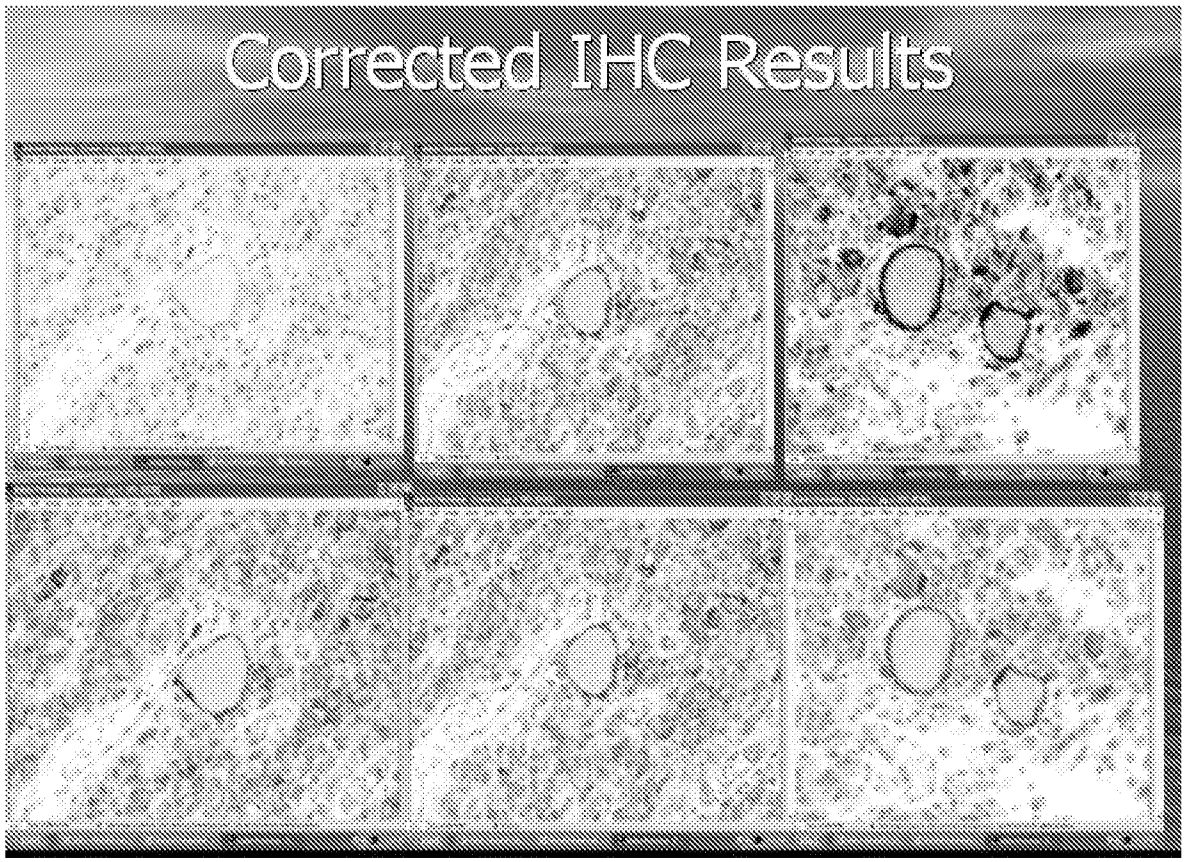

FIG. 7

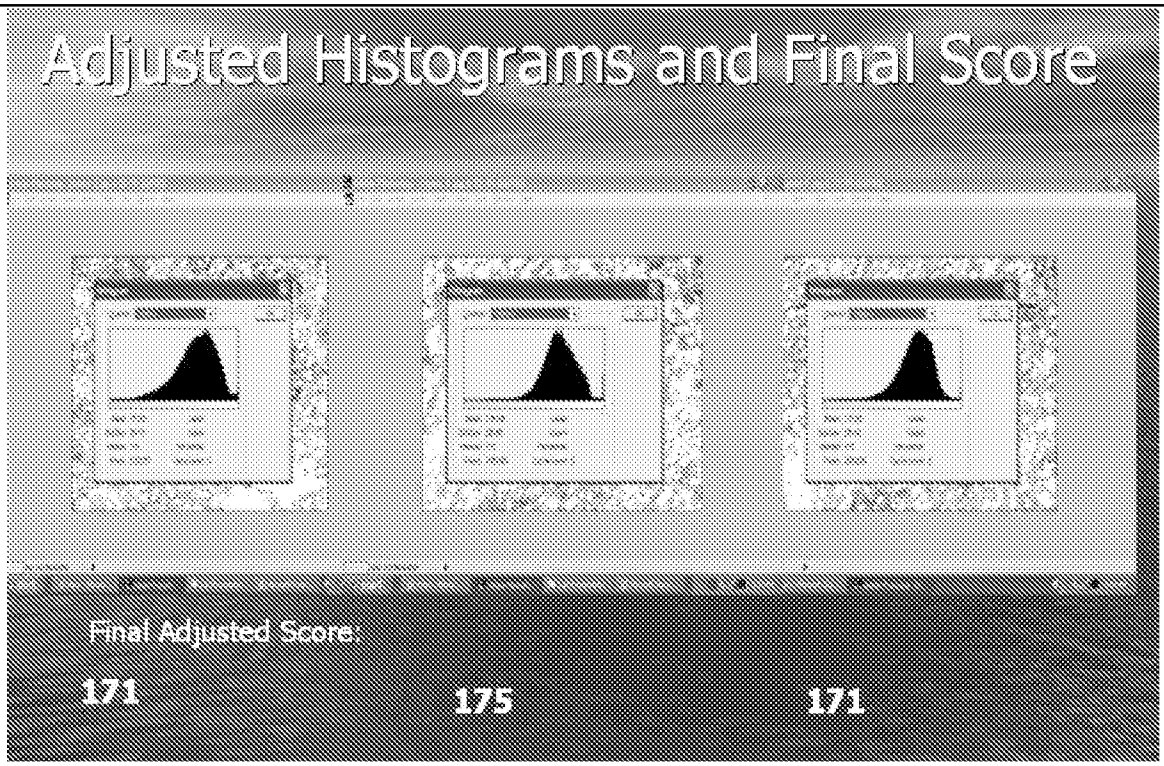
Adjusted Histograms and Final Score
Final Adjusted Score:
171
175
171

FIG. 8