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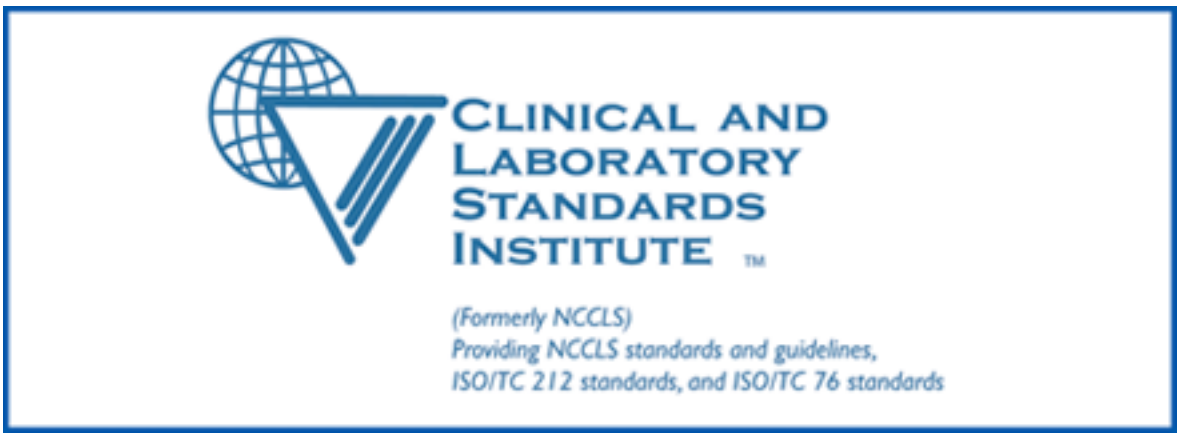
Quality Assurance for Immunocytochemistry; Approved Guideline



This document provides consensus recommendations for the performance of immunocytochemical assays on cytologic or surgical pathology specimens.

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Quality Assurance for Immunocytochemistry; Approved Guideline

Abstract

Prudent clinical use of immunocytochemical assays requires a thorough understanding of the sensitivity, specificity, technical artifacts, and limitations of these assays. Optimal clinical utility further requires that assays be reproducible from day to day and laboratory to laboratory. NCCLS document MM4-A—*Quality Assurance for Immunocytochemistry; Approved Guideline* provides consensus recommendations for the performance of immunocytochemical assays to promote a better understanding of the requirements, capabilities, and limitations of these diagnostic methods; to improve their intra- and interlaboratory reproducibility, and to improve their positive and negative predictive values in diagnosis of disease. This guideline is for use by all clinical and reference laboratories performing immunocytochemical assays on cytologic or surgical pathology specimens, for the manufacturers of commercial reagents and test kits, and for individuals and organizations involved in the development and implementation of laboratory quality assurance programs for these assays.

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Contents

Foreword

This guideline is for use by all clinical and reference laboratories performing immunocytochemical assays on cytologic or surgical pathology specimens, for the manufacturers of commercial reagents and test kits, and for individuals and organizations involved in the development and implementation of laboratory quality assurance programs. The intent of the guideline is to promote a better understanding of the requirements, capabilities, and limitations of these diagnostic methods, to improve their intra- and interlaboratory reproducibility, and to improve their positive and negative predictive values in diagnosis of disease.

The utility of immunocytochemical testing is evidenced by its widespread use throughout the world, and the promise of future utility is demonstrated by the large number of scientific publications proposing new uses for immunocytochemical assays in diagnosis and treatment. In spite of widespread application, however, the number of different immunocytochemical procedures in daily use is probably about as large as the number of laboratories using them. The result has been less than optimal interlaboratory reproducibility, and calls for increased steps to promote uniformity of immunocytochemical assay performance. Nevertheless, few guidelines or standards exist which address the use of immunocytochemical tests as an adjunct method in diagnostic pathology, and those which do exist have not undergone rigorous review by the laboratory community. This document is an attempt to redress this deficiency.

The chosen methods and quality assurance approaches are not absolute or immutable. They represent formal recommendations presented by the subcommittee for consensus review, and thus represent an amalgamation of quality assurance methods currently in use in a large number of laboratories worldwide.

The guidelines described in this document (MM4) are restricted to immunocytochemical procedures and have not been designed to address quality control in *in-situ* hybridization. Many of the principles associated with good immunocytochemistry are also pertinent to *in-situ* hybridization.

Key Words

Antibody, antigen, biopsy, cross reactivity, cytology, immunoassay, immunochemistry, immunocytochemistry, immunohistochemistry, immunomicroscopy, sensitivity, specificity

Quality Assurance for Immunocytochemistry; Approved Guideline

1 Introduction

Proper interpretation and reporting of tissue biopsies and cytologic specimens is critical for the effective treatment of many seriously ill patients. In recent years, the ability to properly interpret these specimens has been enhanced by the availability of immunocytochemical methods for recognizing cellular antigens. These immunocytochemical assays make it possible to more accurately identify tumors whose cytologic and architectural characteristics are not, in themselves, sufficient to enable reliable diagnosis. This in turn makes it possible to more effectively predict the likely response of these tumors to various possible treatments, and to thus optimally treat an affected patient. Other assays make it possible to readily recognize the presence of viruses or microorganisms; yet others identify abnormal gene products. As a result, immunocytochemical assays (immunohistochemical assays) have become an important part of the technical repertoire of the diagnostic pathologist.

Prudent clinical use of immunocytochemical assays requires a thorough understanding of the specificity, sensitivity, technical artifacts, and limitations of these assays. Optimal clinical utility further requires that assays be reproducible from day to day and laboratory to laboratory.

Attainment of such reproducibility requires recognition that quality assurance for immunocytochemical testing starts from the moment the specimen is obtained. This guideline is intended to provide a tool by which clinicians and laboratorians may be assured that immunocytochemical test results are effective and reproducible in aiding diagnosis and guiding therapy. Thus, the guideline covers specimen acquisition and fixation, as well as immunocytochemical assay procedure, interpretation, and reporting. It should thus prove useful to surgery, histology, pathology, and quality assurance staff, as well as to manufacturers of immunocytochemical reagents and those involved with regulation of reagent manufacture or laboratory medicine.

2 Scope

The use of immunocytochemical methods, though well established, nevertheless continues to challenge the pathologist. Despite the clear benefits of having another method by which to gain insight into the nature of disease processes, issues of sensitivity, specificity, and reproducibility mandate the application of stringent laboratory practice. To ensure the success of immunocytochemical diagnostic methods, several key areas warrant attention. This document addresses the following topics as they relate to the use of immunocytochemical methods in diagnostic pathology and laboratory medicine:

- Tissue collection and sample handling
- Fixation
- Post-fixation processing
- "Antigen retrieval" methods
- Immunocytochemical staining protocols
- Automation
- Internal and external controls
- Troubleshooting
- Validation of new immunocytochemical assays
- Reporting of assay results.

The importance of laboratory participation in external quality assurance activities, such as proficiency testing programs, is a given. In this document the subcommittee assumes that the laboratory will take part in such programs, when available. However, it is beyond the scope of this document to suggest the elements required of such external quality assurance activities.

The document should be useful to pathologists, histotechnologists, manufacturers of immunocytochemical devices and reagents, and regulatory personnel.

3 Definitions[a]

In this document, the following definitions of terms are used:

Affinity, *n* – ***In Immunology,*** a measure of the attraction, or force of association, between a single antigenic site and a single antibody to that site.

Affinity constant, K_a, *n* - ***In Immunology,*** **1)** The equilibrium constant for the receptor + ligand reaction. **NOTE:** The average or mean affinity constants are usually described for polyclonal antisera because of their heterogeneity.

Antibody, *n* - **1)** The functional component of antiserum, composed of a population of Y-shaped protein molecules, each member of which is capable of reacting with (binding to) a specific antigenic determinant. **NOTE:** These antibodies are produced by B-lymphocytes as a primary immune defense.

Antigen, *n* - ***In Immunology*** **1)** Any substance that can stimulate the production of antibodies by an organism and combine specifically with them.

Antiserum, *n* - A serum produced in animals or human beings containing antibodies to one or more antigens of interest.

Biotin, *n* - A molecule that can be covalently attached to lysine residues of proteins; **NOTE:** a) The protein avidin has a high affinity for biotin; b) This property can be used in many detection systems.

Blocking, *n* - 1) The reaction of endogenous peroxidase in a tissue specimen with the intent of destroying enzyme activity.

Buffer, *n* - A solution or reagent that can resist a change in pH upon addition of either an acid or base.

Control//control material, *n* - A device, solution, or lyophilized preparation intended for use in the quality control process. **NOTES:** a) The expected reaction or concentration of analytes of interest are known within limits ascertained during preparation and confirmed in use; b) Control materials are generally not used for calibration in the same process in which they are used as controls.

Cross-reactivity, *n* – ***In Immunology,*** the reaction of an antibody with an antigen other than that which elicited its formation, as a result of shared, similar, or identical antigenic determinants.

Diagnostic test, *n* - A measurement or examination of a diagnostic specimen for the purpose of diagnosis, prevention, or treatment of any disease or the assessment of health or impairment of health of an individual patient; **NOTE:** Laboratory tests are often called "*in vitro* diagnostic tests."

Enzyme conjugate, *n* - One of the reagents of an immunoassay that has either an antigen or antibody complexed to an enzyme by a covalent linkage.

Epitope//antigenic determinant//(determinant), *n* - **1)** The minimum molecular structure of the antigenic site that will react with a monoclonal antibody; **2)** Any site on an antigen molecule at which an antibody can bind; the chemical structure of the site determining the specific combining antibody.

False negative result//False negative, FN, *n* - A negative test result for a patient or specimen that is positive for the condition or constituent in question.

False positive result//False positive, FP, *n* - A positive test result for a patient or specimen that is negative for the condition or constituent in question.

Fluorescence, *n* - Brief electromagnetic radiation emitted as a result of absorption of radiation (photons) by an atom, molecule or ion; **NOTE:** Generally, fluorescent radiation has a longer wavelength than the absorbed radiation.

Hybridization, *n* – A base pairing of complementary strands of nucleic acid by hydrogen-bond formation; the binding of probe to specific nucleic acid sequences or amplification products. **NOTE:** Hybridization can be performed with both nucleic acid

[a] Some of these definitions are found in NCCLS document NRSCL8 – *Terminology and Definitions for Use in NCCLS Documents.* For complete definitions and detailed source information, please refer to the most current edition of that document.

target and probe in solution, or with the nucleic acid target retained within a tissue specimen; this latter form of hybridization is referred to as "*in situ* hybridization."

Immunoassay, *n* - **1)** Any laboratory method for detecting a substance by using an antibody reactive with it**; 2)** A ligand-binding assay that uses a specific antigen or antibody capable of binding to the analyte.

Immunocytochemical assay, *n* - An immunoassay which detects an antigen present in a specimen which is contained within intact or histologically sectioned cells or tissues. **NOTE:** Such an assay is also referred to as an "immunohistochemical assay"; the process that encompasses the preparation and examination of tissues stained in this way is variously referred to as "immunocytochemistry," "immunohistochemistry," "immunohistology" or "immunomicroscopy," among others.

Label, *n*- 1) ***In Immunology,*** a substance that is linked to a reagent (e.g., antigen or antibody) to facilitate detection in an immunoassay system using either a measurable property of the label or an entity produced by the label; **NOTE:** In EIA the label is the enzyme; in FIA the label is a fluorescer; in RIA the label is the radionuclide.

Ligand, *n* - An entity that binds to a receptor; **NOTES:** a) For example, an atom, ion, molecule, antibody, hormone, or drug; b) In immunological testing, the terms "ligand" and "analyte" are frequently used synonymously.

Monoclonal, *adj* - Arising from a single clone of cells; **NOTE:** All molecules of a monoclonal antibody have a single class and subclass of both heavy and light immunoglobulin chains, and a single antigenic-determinant specificity.

Nucleic acid, *n* - A polynucleotide made up of dNTPs (DNA) and rNTPs (RNA) linked to one another by phosphodiester bonds, forming macromolecular structures.

Polyclonal antibodies, *n* - A mixture of antibodies derived from different clones and thus having different specificities; **NOTE:** Antiserum produced by immunization of intact animals is usually polyclonal.

Reactivity, *n* - 1) The qualitative assessment of binding of an antigen or antibody with another substance; **NOTE:** Sometimes used as a synonym for "positive" when reporting test results.

Sensitivity*, n* - ***1) In Quantitative testing,*** The change in response of a measuring {system or} instrument divided by the corresponding change in the stimulus; **NOTES:** a) A significant scientific dispute exists regarding this term, its underlying concept and its definition, with the opposing view defining **Sensitivity** in a manner similar to **VIM93**'s[1] definition for **Limit of Detection**. While ordinarily, a **VIM93** citation as given would be sufficient to settle the dispute, a significant case has been made on both sides. Consequently, until the dispute is scientifically settled, the definition above is a standard only if it is clearly stated in the context of its use in a document. b) The sensitivity may depend on the value of the stimulus; c) The sensitivity depends on the imprecision of the measurements of the sample; **2)** ***In Qualitative testing,*** The test method's ability to obtain positive results in concordance with positive results obtained by the reference method; **NOTE:** d) If the true sensitivity of a device is better than the reference method, its apparent specificity will be less and the level of apparent false-positive results will be greater; **3) Clinical sensitivity,** *n* - The proportion of patients with a well-defined clinical disorder whose test values are positive or exceed a defined decision limit (i.e., a positive result and identification of the patients who have a disease); **NOTE:** e) The clinical disorder must be defined by criteria independent of the test under consideration.

Signal//Measurement signal, *n* - A quantity that represents the measurand and which is functionally related to it.

Specificity*, n* - **1)** The ability of a test or procedure to correctly identify or quantify an entity in the presence of interfering phenomena/influence quantities; **2) Analytical specificity,** *n* - For quantitative tests, the ability of an analytical method to determine only the component it purports to measure or the extent to which the assay responds only to all subsets of a specified analyte and not to other substances present in the sample; **3)** For

qualitative or semiquantitative tests, the method's ability to obtain negative results in concordance with negative results obtained by the reference method; **NOTE:** a) ***In Immunology,*** specificity is an antiserum quality defining its reactivity with defined antigens and lack of specificity is the inaccuracy introduced by cross-reacting and/or interfering substances, because cross-reacting substances compete with the analyte for antibody-binding sites; **4) Clinical specificity,** *n* - The proportion of subjects who do not have a specified clinical disorder whose test results are negative or within the defined decision limit.

Titer, *n* - **1)** The reciprocal of the dilution factor required to produce a defined outcome in a defined system; **NOTE:** The titer is usually proportional to the analyte concen-tration; **2)** ***In Radioimmunoassay***, the dilution of the antibody at which a specified percentage of the radiolabeled analyte is bound under defined conditions.

Touch preparation, *n* - A preparation of cells on a microscope slide, prepared by lightly touching a tissue specimen to the slide. **NOTE:** The cells which are thus transferred can then be stained after air-drying or fixation.

4 Safety

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that synthesize the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal pre-cautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (Guideline for Isolation Precautions in Hospitals, Infection Control and Hospital Epidemiology, CDC, Vol 17;1:53-80.), [MMWR 1987;36 (suppl 2S):2S-18S] and (MMWR 1988; 37:377-382, 387-388). For specific pre-cautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*.

Many of the solvents and chemicals used in immunocytochemistry are toxic, flammable, or both. For example, two commonly used chromogens, diaminobenzidine (DAB) and aminoethylcarbazone (AEC), have both been reported to be carcinogenic. Xylene, toluene, acetone, and ethanol are all highly flammable. Laboratorians performing immunohistochem-ical tests should have access to, and should regularly review, the *Material Safety Data Sheets* for all chemicals used in the laboratory. Guidelines for waste disposal are provided in the most current edition of NCCLS document GP5 — *Clinical Laboratory Waste Management*.

Handling of hazardous materials is highly regulated by many government agencies. Laboratory workers should be aware of, and scrupulously adherent to, the various laws and regulations pertinent to the chemicals used in the immunocytochemistry laboratory.

5 Tissue Sampling and Processing

5.1 Sample Collection

The following should be considered by the surgeon prior to collection of tissue samples:

- Preoperative consultation with pathology staff (as needed) to plan selection of most representative site(s) to establish diagnosis by histopathologic techniques and requirements for selection, handling, transporting, fixation, and processing of tissues.

- Size of biopsy:

  — Perform excisional biopsy or resection whenever possible (standard surgical clinical practice).

    - Remove entire tumor intact (ideal).

    - Ensure that surgical margins include contiguous normal tissue.

— Perform incisional biopsy when a tumor is too large to remove intact, is in an inaccessible site, or a follow-up resection is planned.

  - Multiple samples may be necessary to ensure adequate representation of the tumor and to characterize the most advanced grade or anaplasia of the tumor.

  - Normal tissues in margins of the tumor specimen that are contiguous with abnormal tissue can serve as internal control tissue for the entire immunocytochemical (ICC) processing and interpretation (normal blood vessels and stroma within the tumor may serve as internal control tissue for some antigens).

5.2 Sample Handling

5.2.1 Sample Handling by the Surgeon

It is standard clinical practice that the surgeon does not slice the tumor specimen in the operative suite. Examination of the biop-sy specimen is delegated to the pathology staff for a timely evaluation of the gross tissue specimen, selection of samples for microscopy, and histopathologic diagnosis.

Although neutral-buffered formalin is an appropriate fixative for most immunocytochemical assays, certain immunocytochemical assays, molecular assays, as well as electron microscopic examinations are best performed on tissue fixed in substances other than formalin. It is often useful, therefore, to fix specimens in more than one fixative. Preoperative communication between operating room staff and the pathologist is useful in assuring that tissue is optimally preserved to facilitate all appropriate diagnostic tests.

5.2.2 Sample Handling by the Surgeon s Assistant

Consult with pathology staff prior to surgery for instructions about how to handle a specimen before it is received by pathology staff. Pathology staff should guide collection and handling according to the most likely target antigens that will be studied. The following points should be considered in handling tissue samples:

- Antigens vary in their stability and need for immediate or rapid preservation by freezing (e.g., snap freezing of estrogen receptors in liquid nitrogen within five minutes of excision if, estrogen receptors (ER)/progesterone receptors (PR) immunocytochemical assays are to be performed on frozen, rather than paraffin-embedded tissue).

- Usually tissue should be placed directly into a clean, dry container for timely transportation to the location used for gross tissue examination.

- Tissue may be placed in physiologic saline, Ringer's solution, or other solution on gauze or submerged in solution. Some physiologic saline is not physiologic; it has a pH of 5.5 and may cause damage to cell morphology. Air drying is a problem if processing for frozen section and/or fixation are delayed.

- Ensure that all specimen containers and slides are labeled properly. Proper identification of the patient from whom the specimen is obtained, the type of specimen, and the time of collection are essential and should be required for all specimens accepted for testing.

- Biologic hazards precautions should be followed. (See the most current edition of NCCLS document M29— *Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*.)

- Reserve some of the tissue samples for ancillary testing as follows:

  — Nondestructive testing:

    - Touch preparations from the cut surface of the biopsy are usually handled as cytopathology specimens, although they are suitable

for ICC staining if a procedure has been established on this type of specimen and fixation.

- Microbiologic samples may be required. Plan ahead as to whether the targeted microorgan-ism antigens will require fresh or formalin-fixed tissue specimens for ICC analysis and whether culturing or other methods will be required.

— Gene amplification or rearrangement by nucleic acid methodology can be nondestructive (e.g., fluorescent *in situ* hybridization) or destructive (e.g., Southern blot hybridization).

It is important that specimens be transported to the pathology laboratory as soon as possible to facilitate prompt gross examination, dissection, fixation, and embedding.

5.2.3 Sample Handling by the Pathology Staff

Frozen sections for conventional histopathology should be handled by the pathology staff. Gross inspection and selection of the most diagnostically representative area is reserved for the conventional histopathologic diagnosis. Residual tissue is retained in frozen state for immunocytochemistry staining with reagents that require frozen tissues, because they do not work with fixed and/or processed tissue.
After the histopathologic diagnosis is made, the residual frozen tissue is processed for paraffin embedding for conventional histopathology, with or without immunocytochemistry, with reagents having previously demonstrated functionality with formalin-fixed, paraffin-embedded tissue sections.

5.3 Fixation

5.3.1 Standard Fixation

The use of fixatives and means of fixation is likely the most variable of factors which can affect the outcome of ICC analysis. Conditions of fixation (i.e., 1] type of fixative employed; 2] date and time placed into fixative; and 3] total fixation time) should be noted for each tissue sample to be analyzed.

Routine paraffin-embedded tissue is subjected to fixation twice: at the time of excision and again on the tissue processor. If the initial fixative is different from the fixative on the processor, know the potential impact and compatibility of this difference. The initial time in the fixative plus the additional duration of time on the tissue processor should be considered when assessing overall fixation times.

Standardization of fixatives, especially fixation times, is difficult within and between institutions due to the variability in surgical procedures and tissues. (For the subcommittee's rationale regarding the need for 'standardization' of various steps in the immunostaining and tissue fixation process, refer to Comment 5 in the Summary of Proposed-Level Review Comments and Subcommittee Responses.) It is therefore important to monitor the conditions of fixation such that the potential for detection of false positives or false negatives, which might be attributed to elements of fixation, can be optimized. The laboratory should establish the performance characteristics of the fixatives used.

The choice of fixative influences the need for pretreatments, titer of antibody required, and the background and intensity of staining. Some fixatives are not compatible with certain antigens/antibodies. Further, under- or overfixation can interfere with immunoreactivity. The fixative and treatment used for control tissue samples should be identical with that used for patient tissue samples.

5.3.2 Microwave Fixation

5.3.2.1 General Considerations

Microwave applications can potentially greatly reduce the amount of time necessary for processing and staining. The following should be considered when performing microwave fixation:

- **Warning: <u>Only</u> approved laboratory microwave ovens should be employed for laboratory procedures.** This is a safety issue; the use of normal "kitchen" microwave ovens that are not properly vented can result in explosion and/or serious operator injury or illness. Microwave ovens should be dedicated to laboratory procedures only. At no time

and under no circumstances should the oven be used for heating foods or beverages.

- **Warning: Never place metal objects in the microwave. Applications should be carried out in plastic coplin jars or humid chambers.**
- Necessary exposure times vary with the make and model of microwave oven employed, the amount of power output, and the type of staining jar or humid chamber used. In most units, the maximum flux for microwave energy is within a 6-cm radius of the oven center. Whenever possible, slides/tissue should be placed within this area, regardless of whether the microwave oven is equipped with a carousel or is a noncarousel model.
- **Warning: Solutions and slides will get hot in microwave applications.**
- **Warning: Certain vapors (e.g., from formalin or Bouin solutions) are volatile. It is recommended that the microwave be placed in a fume hood, and the microwave door should be left open between procedures. Vented microwave ovens designed for the laboratory are commercially available and should be the microwave of choice.**
- **Warning: Cover jars/dishes/chambers loosely or drill holes in the lids.Tightly closed containers may explode.**
- Tissues immersed in conventional fixatives (e.g., formalin, ethanol-polyethylene glycol [EtOH-PEG], methanol-polyethylene glycol [MetOH-PEG]) for abbreviated times at room temperature demonstrate generally uniform fixation and staining integrity throughout the tissue when subsequently subjected, while in fixative, to microwave exposure.
- Tissue specimens should be no more than 5 mm in thickness.
- Specimens should be immersed, at room temperature, for a minimum of four hours in formalin, one hour in EtOH-PEG, or 45 minutes in MetOH prior to microwave treatment.
- For a 5-mm thick specimen, immerse in not more than 100-mL fixative in a microwave-compatible container. Cover loosely. Microwave for 5 minutes or less. For example, sufficient fixation is achieved with a 5-mm specimen in formalin by microwaving in a 450-watt oven for 1.5 to 4 minutes at 55 °C.

5.3.2.2 Temperature Measurement

Standard volume solutions can be heated and the temperature measured as a form of monitoring oven temperatures. However, it should be recognized that the temperature of solutions heated and the "read-out" temperature of the oven may vary by as much as 15 to 30 °C.

Standard or laboratory fluid thermometers should *not* be used for temperature measurement inside the microwave. These will readily explode when subjected to microwave heating. Special commercially available microwave transparent fluid thermometers can be used for this purpose.

An attempt should be made to establish temperatures at different times and power levels for fixation. These temperatures should be charted and monitored as part of the normal laboratory instrument quality control procedure. Generally, temperatures within the oven should not exceed 60 °C. Temperature control can be critical for consistent results in immunocytochemical staining.

The length of time necessary to achieve a certain temperature is directly proportional to the load of the oven (e.g., the amount of tissue, fixative, or number of slides present). The use of self-contained staining chambers (e.g., humid chambers) is recommended. Local temperature control may be more easily achieved in this type of vessel.

5.3.3 Fixatives

Table 1 provides a comparison of the tissue fixatives described below.

5.3.3.1 Formalin

Formalin is the most widely used fixative in histopathology. Formalin fixatives are believed to best preserve cell and tissue structure. Commercially available formalin stock solutions are usually 37% to 40% formaldehyde in water. These may be diluted

1:9 in water or buffer to achieve a 10% formalin (4% formaldehyde) solution. Para-formaldehyde may be dissolved to 4% w/v in water or buffer (in a well-ventilated hood) while heating at 60 °C. Dissolution is slow and may be assisted with the addition of a few drops of 1Mol/L NaOH. Conventional fixation is usually carried out in 10% neutral buffered formalin (NBF). NBF is commercially available in a ready-to-use form. Although formalin penetrates the tissue quickly, actual fixation is slow. Fixation in formalin requires a 1:10 specimen to fixative ratio and times of greater than 8 hours, with 24 to 48 hours necessary for complete fixation. The total amount of fixation time should be standardized.

Long-term storage in formalin is possible, or in 70% ethanol following fixation in NBF. Long-term storage in formalin is best applied for routine histopathology but may not be acceptable for ICC procedures. Formalin in specimen containers should be replaced weekly, and a standard pH should be adopted, either neutral or slightly acidic.
Although widely used, NBF may not be the fixative of choice for ICC procedures.[2] Reports have indicated variable adverse effects of NBF fixation, especially resulting from long-term storage, for the performance of ICC procedures. Specimens fixed or stored in NBF may be harder to process and can result in antigen cross-linking, leading to the need for antigen retrieval, and/or partial or irretrievable antigen disappearance.

5.3.3.2 Zinc Formalin

Zinc formalin fixation has demonstrated equivalent preservation and better recovery of ICC reactivity. Zinc formalin fixatives are commercially available. Fixation times can be shorter than with NBF, 4 to 48 hours, with as little as 4 to 6 hours resulting in good fixation. Improved ICC staining has been shown with zinc formalin and has minimized the need for antigen unmasking or retrieval. Zinc formalin has been reported as potentially quenching primary fluorescence when tissue is evaluated by fluorescent microscopy.

5.3.3.3 Alcohol/Acetone

Immersion in 70% or 95% ethanol may also provide adequate fixation for tissue, but may lead to a decline in the quality and integrity of ICC staining, especially following long term storage. Ethanol can, itself, have a shrinking and hardening effect on tissue. Tissue incompletely fixed by NBF or zinc formalin will be subjected to alcohol fixation in the tissue processor. Fixation of cryostat frozen sections for five to ten minutes in ambient or cold acetone or a 90% EtOH to 10% acetone mixture can provide suitable fixation.

5.3.3.4 Other Fixatives

Fixatives such as Bouin, B-5, glutaraldehyde, acrolein, etc. have applications which may give varied results, and limited compatibility with some systems, e.g., tissue processors. Alternative fixative mixtures have also been employed with varied success, such as ethanol or methanol/polyethylene glycol (PEG) and formaldehyde/glutaraldehyde.

Table 1. Comparison of Tissue Fixatives

Fixative	Description	Suggested Fixation Times*	Reported Benefits	Reported Disadvantages
Formalin	10% neutral buffered formalin	>8 hrs., 24-48 hrs. often necessary for complete fixation	most common fixative; readily available, penetrates tissue quickly	fixation is slow, may not be complete with shorter times; may not be suited to long-term storage of tissue for ICC; harder specimens; antigen (Ag) cross-linking often results with need for Ag retrieval; partial or irretrievable Ag disappearance possible; special handling and disposal requirements
Zinc Formalin	mixture of zinc sulfate and formalin	4-48 hrs., as little as 4-6 hrs. for complete fixation	shorter fixation times; minimal need for antigen unmasking or retrieval; preserves better tissue Ag morphology	possible quenching of primary fluorescence; special handling and disposal requirements
Alcohol/Acetone	70% or 95% EtOH; 90% EtOH/10% Acetone	variable; often occurs in tissue processing secondary to formalin fixation; 10-15 minutes for cryostat sections/cytology smears	shorter fixation times; better cryostat sections (cold EtOH/acetone); good preservation of cytoplasmic intermediate filaments	decreased quality/integrity of ICC staining; shrinking and/or hardening of the tissue
Bouin	mixture of formalin and picric acid	1-12 hrs.	fixes tissues rapidly; useful particularly for endocrine tissues and tumors	decreased preservation of many types of Ags, particularly lipid-containing Ags; longer fixation may result in “brittle” tissues; poor penetration may result in under-fixation; special handling and disposal requirements
B-5	mixture of mercuric chloride, sodium acetate, and formalin	1-6 hrs.	primary use in lymph node tissue; enhanced cytologic detail and immunoreactivity of cytologic immunoglobulin (Ig), intracytoplasmic Ags.	tissue hardening; surface Igs not well demonstrated; special handling and disposal requirements

*Suggested fixation times are quite variable and are based on type of tissue, tissue thickness, etc.

A fixative containing ethanol and polyethylene glycol is generally substituted for formalin. Tissues of 5 mm^3 are generally fixed within four hours at ambient temper-atures. The presence of PEG in the solution occasionally minimizes the adverse effects of the ethanol. Good, clear ICC staining has been reported with this fixative. Some types of tissues fixed with ethanol and polyethanol glycol may show tissue membrane detach-ment and more shrinkage of the tissue than routinely observed with formalin fixation. However, antigen unmasking and retrieval are usually unnecessary with this fixative.

5.4 Postfixation Processing

5.4.1 Xylene

Refer to the most current edition of NCCLS document GP15—*Papanicolaou Technique*, for recommendations for handling xylene, and precautions for conventional staining.

5.4.2 Xylene Alternatives

The pathologist should complete a comparison study to verify the suitability of processing ICC specimens with xylene alternatives, by using paired fresh samples that are identical, with the exception of xylene vs. xylene alternative processing. See also the most current edition of NCCLS document GP15—*Papanicolaou Technique,* for recommendations for handling xylene alter-natives.

5.4.3 Decalcification

Decalcification, whether performed using acid or chelating agents, may reduce the reliability of subsequent immunocytochemical assays. Antigen retrieval procedures may not restore the detectability of these antigens. The ability to detect intermediate filaments, such as keratin, may be preserved when the ability to detect other antigens has been lost. The time to which tissue is subjected to decalcifying agents should be minimized, and results of immunocytochemical assays on decalcified tissue should be interpreted with caution reflecting the possibility of antigen loss.

5.5 Sample Processing and Handling

5.5.1 Paraffin[3]

5.5.1.1 Dehydration and Clearing

Dehydration removes the water from within the tissue and cells. Clearing agents subsequently remove the dehydration agent from the tissue. Adequate dehydration and clearing are both necessary in the preparation of tissue for paraffin embedding. Paraffin will not enter the tissue if it contains water. Partial dehydration leads to improper clearing, which can leave the consistency of the tissue "soft and mushy."

Various forms of alcohol are employed for dehydration. Acetone may also be used, but can cause excessive shrinkage of the tissue, as well as posing increased flammability and volatility hazards in tissue processors.

Standard processing includes processors employing general progressions that increase the concentration of the dehydrant, e.g., 60% alcohol --> 65% alcohol --> two changes of 95% alcohol --> absolute alco-hol, with 15 minutes to no more than 3 hours in each change, depending on the type and size of the tissue, and individual processor efficiency.

Xylene is the most commonly used clearing agent, but should be handled with the precautions outlined in Section 5.4.1. Tissues should not be left in xylene for prolonged periods, because this tends to over harden the specimen. Xylene has also been reported to increase background staining. Toluene may be used for clearing, and is reported to not have the overhardening effect of xylene. Chlo-roform may also be used for clearing, but penetrates the tissue more slowly, thus prolonging the clearing process.

The tissue is usually incubated in two changes of the clearing agent, for between 15 to 60 minutes in each change, depending on the type and size of the tissue, extent of dehydration, and individual processor effi-ciency.

5.5.1.2 Embedding

The following should be considered when embedding tissue in paraffin:

- Due to the possibility of excessive heat inactivation of tissue antigens, it is best to use paraffins of lower melting points (e.g., 55 to 58 °C). Differences in the quality of the embedded tissue can vary with the variable melting point of different types of paraffin. Further, infiltration time (i.e., the time the tissue is kept in hot paraffin) should be kept at a minimum. Excessive heat and exposure to the paraffinization process can, in addition to adversely affecting antigens, cause tissue shrinkage and hardening.

- Two to three changes of paraffin should be used, ranging from 15 to 90 minutes in each change, depending on the individual tissues. A progression (e.g., 30 minutes --> 60 minutes --> 90 minutes) is recommended.

- Close quality control should be maintained on paraffin. Temperatures of the paraffin should be monitored and recorded daily. Because clearing agents can contaminate paraffin, frequent rotation and replenishment of the paraffin should be practiced.

- Agents such as a paraffin in combination with plastic polymers have been suggested to lower sensitivity in peroxidase-antiperoxidase methods.

- Tissues should be flat and diagonally oriented in the mold, rather than parallel with the mold edges. Proper tissue orientation is dependent on the type of tissue being processed.

- If reagent containers are low or empty for any portion of the processing, the tissue specimen may not be completely immersed and may dry out.

- Tissues properly fixed and embedded may be kept indefinitely if stored in a cool place. Satisfactory immunoreactivity of tissues should be verified by the user.

5.5.1.3 Sectioning

Either steel or disposable knives are acceptable for sectioning paraffin. It is essential to keep knives sharp and free of defects.

The following steps should be performed when sectioning tissue samples:

(1) Set knife angle to 30° (from center line of the knife to the block face). Adjust angle from 30° if sectioning difficulties arise. Angles greater than 30° may result in scraped sections; angles less than 30° may result in sections that are compressed or vary in thickness.

(2) Check working condition of microtome. Microtome should be thoroughly cleaned daily with solvent to remove buildup of excess paraffin. Oil instruments according to manufacturer's recommendations and cover them when not in use.

(3) Set cutting thickness of the microtome, as described by the manufacturer, at 6 µm or less. Optimal tissue section thickness may vary depending on the type of tissue being processed.

(4) Position a paraffin-embedded tissue block on the instrument and lock into place.

(5) Release the drive wheel and advance the tissue sample to nearly contact the knife edge. Depending on the type of paraffin used, adjust the tissue block to the desired temperature with a rapid-freezing reagent.

(6) Rough-cut the tissue sample to obtain the desired cutting plane. Clear the knife of any debris.

(7) Adjust the block to the desired temperature with a rapid freezing reagent.

(8) Turn the drive wheel with even, smooth, consistent motion to obtain a sectioning ribbon. Grasp the paraffin ribbon gently with forceps and lay out onto the flotation

water bath. Temperature of the water bath is normally 5 to 10 °C below the melting point of the paraffin used. Excessively hot water baths will result in tissue artifacts.

(9) The paraffin ribbon should be gently stretched as it is laid out on the water. Quickly and gently "tease" out any wrinkles or folds. Transfer paraffin ribbon from the knife to the water bath and remove wrinkles and folds.

(10) Place a clean, pretreated (see Section 6.2), room-temperature glass slide into the water bath at a 45° angle and bring it up and under the paraffin section. Lift the section out of the water with the glass slide. Care should be taken not to disrupt any air bubbles that have formed on the bottom of the water bath, since air bubbles trapped beneath the section prior to drying may cause staining artifacts.

(11) Dry mounted sections completely prior to deparaffinization. Sections can be air dried at room temperature for 24 hours, dried in an oven, with a hot air blower, on a slide plate warmer, or any other conventional drying apparatus. Some devices (particularly slide drying plates) used to dry slides demonstrate wide temperature swings. Care should be taken that the temperatures of these devices do not exceed 60°C (with resulting antigen degradation) at any time.

5.5.2 Frozen Sections

5.5.2.1 Freezing

Various methods can be employed for preparing frozen blocks of tissue, e.g., snap freezing in liquid nitrogen, on dry ice, in cold isopentane, in cold acetone, in the cryostat, or with a CO_2 jet. Snap freezing is highly recommended. Slower freezing processes (e.g., in the cryostat) can lead to the formation of ice crystals in the tissue and subsequent artifact in staining, as well as being less likely to preserve some antigens (e.g., estrogen receptors). The rate of freezing associated with the use of hydrochlorofluorocarbon refrigerant spray bottles is difficult to control. Blocks can be prepared in commercially available plastic molds designed for this purpose or by preparing approximately 2 to 3 cm molds of aluminum foil.

Liquid nitrogen is dangerous when spilled or splashed. It should be handled carefully with protective clothing and other protective equipment (e.g., goggles to protect eyes and thick gloves to protect hands.)

When using liquid nitrogen for preparing tissue for frozen section, a small amount should be placed in a suitably sized flask for current/daily routine use. After use, this must be discarded safely and NOT be returned to the stock storage container.

The following steps should be performed when freezing tissue samples:

(1) Place a layer of embedding medium into the mold, and immerse the mold into the freezing medium with a long pair of tongs or forceps. Embedding medium should freeze in 15 to 20 seconds. Plastic tongs and cold-insulated gloves should be used whenever possible. Build up another layer of embedding medium, and make sure it solidifies slowly. The building up of layers of embedding medium assures ample clearance during cutting. Layering is accomplished by keeping the medium solid at the bottom but liquid at the top until the embedding of tissue is completed. The exposed edge of tissue should be protected with additional embedding media to avoid freezer burn during long-term storage.

(2) For long-term storage, one may attach a prelabeled cork disk to the mold with embedding medium, then use additional embedding medium to attach the tissue to the cork disk. This provides a conveniently attached label, and an object by which to manipulate the tissue without actually touching it with the forceps.

(3) Using fine-tooth forceps, pick up tissue at the periphery to avoid crushing artifacts. Blot free of any excess moisture within the tissue. Place the tissue in the center of the liquid portion of embedding medium (proper orientation of the tissue is determined by the tissue type), cover with more embedding medium, and slowly sub-

merge into the freezing medium for a snap freeze. Avoid bubbling of embedding medium. Cracking of the block may be avoided by immersing the mold for about five-second intervals and removing it for one to two seconds between intervals.

(4) Blocks should be immediately placed into the freezer, cold cryostat, or onto dry ice. Tissues that are properly frozen may be stable indefinitely. Satisfactory immunoreactivity of fresh or stored tissues should be verified by the user.

(5) Tissue placed into the block should be kept about 5 mm^3 in size.

If a block is to be stored frozen it is convenient either to arrange it for freezing on a cork disc (or several layers of damp filter paper) that can be labeled on the nontissue side with ball-point pen before freezing, or else to mount a prelabeled cork disc on top of the mold before freezing, so it is attached to the block by the embedding medium. The cork is an insulating layer that can be handled with forceps without danger of touching the tissue and can be frozen onto the precooled cryostat object holder with a drop of embedding medium. It can be removed with a razor blade (or by heating the object holder, if the cryostat has a specimen holder that is thermoelectrically controlled), and the frozen embedding medium can be scraped off to reveal the written label.

5.5.2.2 Sectioning

The following steps should be performed when sectioning frozen tissue sections.

(1) Use alcohol- or acetone-cleaned, adhesive-coated slides (see Section 6.2 on slide adhesives) for frozen sections. Sections should be even and flat and cut at 4 to 6 µm. It is recommended upon cutting that sections be immediately fixed in cold (dry ice) acetone for five to ten minutes and placed in a slide box in the cryostat prior to subsequent storage in the freezer, if not used immediately.

(2) Set the knife angle to 30° (from the center line of the knife to the block face). Adjust angle from 30° if sectioning difficulties arise. Angles greater than 30° may result in scraped sections and angles less than 30° may result in sections that are compressed or vary in thickness.

(3) Adjust the cryostat for proper temperature (-10 to -20 °C, depending on the tissue type).

(4) Set the cutting thickness of the microtome to 4 to 6 µm, as described by the manufacturer.

(5) Blocks should be placed in the cryostat prior to sectioning to match cryostat temperature. If not embedded in a block which is embedded on a cryostat object holder, place enough embedding medium onto the object holder to completely cover the surface, and firmly press the frozen block, while in the cryostat, onto the holder. The embedding medium should freeze in 15 to 20 seconds.

(6) Transfer the tissue on the object holder to the cryostat. Be certain the microtome drive wheel is in the locked position before trying to insert the object holder. Position the object holder correctly and tighten according to the manufacturer's recommendations.

(7) Release the drive wheel and advance the tissue sample to nearly contact the knife edge.

(8) Rough cut the tissue sample to obtain the desired cutting plane. Clear the knife of any debris.

(9) Turn the drive wheel until the leading edge of the tissue sample begins to cut. Cutting with an even, smooth, consistent motion, use the camel hair brush to lay the tissue section onto the microtome knife as the drive wheel is simultaneously turned to complete the cut. The "anti-roll" device may be used instead of the camel hair brush. Proper adjustment is necessary to avoid potential sectioning difficulties.

(10) Hold a clean, room-temperature glass slide a short distance (e.g., 2 to 3 mm) from the section on the knife to mount the

section. The warm slide will attract the cold section. Do not use pressure to mount the section. Pressure will cause distortion or create artifacts. Use of adhesive slides will assist in reducing the risk of tissue loss during subsequent staining procedures.

(11) Mounted sections should be fixed in cold (-20 °C) acetone for two to five minutes. These may be stained immediately or stored following fixation by air drying at room temperature and then stored at -70 °C. For some antigens (estrogen and progesterone receptors) buffered formalin acetone (pH 6.6) may give superior results.

(12) Partially cut blocks can be kept for longer periods of time for re-evaluation by re-covering the exposed tissue with embedding medium, snap freezing the embedding medium, and wrapping the entire block in plastic wrap or parafilm. The block is then sealed inside a plastic bag and stored at ultracold temperatures (lower than -70 °C).

5.5.3 Cytologic Specimens

5.5.3.1 Specimen Acquisition and Fixation

Cytologic specimens, whether derived from touch preps, smears, body fluids, or fine-needle aspiration, are excellent substrates for immunocytochemical evaluation. Since enzymatic pretreatment and other "antigen retrieval" techniques can cause these specimens to "wash off" during processing, use of meticulously clean slides pretreated (see Section 6.2) to prevent specimen loss is recommended even more strongly than for other specimens.

Although cell blocks prepared from body cavity fluids are typically fixed in buffered formalin, most other cytologic specimens are fixed in ethanol or ethanol-based fixatives. The pathologist interpreting the immunocytochemical staining pattern must keep in mind the differences in staining associated with different fixatives.

5.5.3.2 Specimen Staining

Cell blocks prepared from body cavity fluids may typically be stained using the same protocols employed with other formalin-fixed, paraffin-embedded specimens. Other specimens frequently require variation in staining protocol. Most cell surface antigens require shorter staining periods for cytologic than for surgical pathology specimens, for example. It is recommended that each laboratory validate its protocols for cytologic specimens separately from those for surgical specimens.

Immunocytochemical stains may generally be applied to Pap-stained specimens following coverslip removal. No change in the staining protocol is typically required.

5.5.3.3 Interpretation

Although the interpretation of immunocytochemical stains is generally the same for cytologic specimens and surgical specimens which have been similarly fixed, some caution is called for when interpreting staining patterns from body cavity fluids. Metastatic tumors growing in these fluids resemble, to some extent, those grown in tissue culture media. Aberrant antigen expression appears to be more frequent in body fluids than in other cytologic specimens.

5.5.4 Electron Microscopy

Immunocytochemical assays may be performed on specimens prepared for electron microscopic examination. Procedures and quality assurance measures for "immunoelectron microscopy" are beyond the scope of this document.

5.6 Processing Problem Determination

5.6.1 Types of Processing Problems

The following are types of processing problems that may be encountered:

- Partial fixation (generally fixed < 24 hours) producing heterogeneity of immune reactivity, with areas of absent reactivity.

- Nonuniform fixation between coagulating and cross-linking fixatives leading to a heterogeneity of detectable immunogenicity throughout the tissue.

- Overfixation (generally fixed >72 hours) producing a decrease or complete loss of immune reactivity.

- Excessive temperature during fixation or processing producing a progressive loss of antigen and immune reactivity.

6 Prestaining Considerations

6.1 Antigen Retrieval

The term "antigen retrieval" initially was applied to the use of microwave heating for enhancement of antigenicity in formalin-fixed, paraffin-embedded tissues. In general, usage of the term "antigen retrieval" now describes a variety of techniques that may be applied when performing immunocytochemical staining on paraffin sections that have been less than ideally fixed.

Heating methods other than a microwave oven, including autoclaving and steam heating, may be applied with equal effectiveness. Terms such as Heat Induced Epitope Retrieval (HIER) or "thermal pretreatment" have been offered as alternative nomenclatures. The extensive literature that has developed around the term "antigen retrieval" weighs against any change in nomenclature, in addition to which the term "antigen retrieval" is scientifically accurate. In a broader sense the term also includes enzymatic digestion, which may be preferable to heat-induced antigen retrieval for some antigens.

The use of a standardized antigen retrieval protocol may frequently, but not invariably, enhance the quality of immunohistochemical staining in tissues for which fixation has not been optimal. (For the subcommittee's rationale regarding the need for 'standardization' of various steps in the immunostaining and tissue fixation process, refer to Comment 5 in the Summary of Proposed-Level Review Comments and Subcommittee Responses.) Variables in the antigen retrieval process that require standardization include the choice of retrieval solution, pH, temperature, time, and heating method. Of several heating methods thus far described, (i.e., conventional heating, microwave heating, pressure cooking, steaming, and autoclaving), all may give equivalent results if temperature and time are adjusted to optimal values. The microwave, pressure cooker, and steamer are easiest to use, but the final choice for each laboratory may be made based upon availability of equipment and convenience. Once a heating method has been selected, each antibody should be titrated to determine the optimal staining and retrieval conditions for the corresponding antigen, for tissues processed in that laboratory. A simple test panel may be employed for this purpose, varying the pH and heating time to achieve maximal retrieval (i.e., the most intense specific staining with the least background staining); employing one of the more commonly used retrieval solutions such as citrate buffer (0.01M, pH 6.0), Tris-HCl buffer (0.01M, pH 9.0) with or without 5% (w/v) urea, or 1mM EDTA-NAOH (pH 8.0)[b]. An example of this test battery approach is shown in Table 2.

Immunocytochemical staining may frequently be performed on previously stained cytologic material (whether stained by Pap or other methods). Care should be used when subjecting cytologic specimens (smears) to antigen-retrieval techniques, since the harsh conditions employed may result in loss of some or all of the tissue cells; loss of tissue or destruction of its architecture may also occasionally occur when antigen-retrieval techniques are employed for underfixed tissue specimens. In addition, the use of antigen-retrieval techniques may sometimes result in increased nonspecific staining, such as increased detection of endogenous biotin.

[b] Dropping 1M sodium hydroxide into 1mM EDTA to reach pH 8.0.

Table 2. Test Battery for Optimal pH & Heating Time (Using choice antigen retrieval solution* and selected heating method†)

pH	Heating time intervals		
	5	10	20
1			
6			
9			

*Usually citrate buffer or Tris-HCl (±5% [w/v] urea).

†Based upon equipment availability and convenience (e.g., microwave, pressure cooker, steamer, autoclave).

The same "battery" approach may be employed to select preferred heating methods or preferred antigen retrieval solution by holding other variables (such as pH and time of heating) constant. The use of slide adhesives is recommended. In addition, do not allow slides to dry out during microwave staining; this can lead to background artifacts.

6.2 Slide Adhesives

The conditions of ICC staining and processing (e.g., proteolytic enzyme treatment for antigen retrieval, long incubations, washing, microwave treatment) tend to be harsh on tissue sections and loosen sections from slides. Therefore, adhesive-coated slides, or commercially available "charged" or "frosted" (not to be confused with the "frosting" at the end for labeling) slides, are recommended for most immunocytochemical techniques.

Many different slide adhesives are available, and the individual use is largely a matter of personal preference and variation in laboratory procedures. These include silane, poly-L-lysine, albumin, gelatin, white glue, and chrome alum. Protein-based adhesives (e.g., albumin) can increase nonspecific background staining. The adhesives of choice for most applications are silane and poly-L-lysine. Use of silanated or poly-L-lysine-coated slides is particularly effective in ICC procedures, especially in microwave use. Solutions of silane and poly-L-lysine are commercially available for coating slides, as are slides precoated with these compounds.

6.2.1 Silane

Slides to be coated with silane should first be cleaned by racking and soaking in clean acetone, then air dried. Prepare a mixture of 250 mL acetone and 5 mL of 3-aminopropyltriethoxysilane. Soak slides for at least two minutes in this solution. Rinse well using two changes of distilled water and dry for 30 minutes at 60 °C. Store in slide box at room temperature.

6.2.2 Poly-L-Lysine

Prepare a 0.01 to 0.1% solution of poly-L-lysine hydrobromide in distilled water. Store the working solution of poly-L-lysine at 4 °C. Filter immediately prior to reuse. Generally, poly-L-lysine compounds of molecular weight (MW) >70,000 are best for tissue adhesion, with optimal results usually observed in compounds of MW of 150,000 to 300,000. Immerse slides in the solution of poly-L-lysine for at least five minutes, drain, and dry overnight at room temperature or for one hour at 60 °C. Store in slide box at room temperature.

As an alternative, poly-L-lysine may also be spread in a thin layer on a slide as for a blood smear. Care must be taken to get a thin enough layer (interference colors seen) by pushing a droplet (10 microliters or less) of the solution along the slide surface with the edge of another slide held at about 30 degrees to the first. Slides are ready for use immediately and can be stored indefinitely.

7 Immunostaining

7.1 Variable Methods

Actual staining methodologies (specific procedure, incubation time and temperature, etc.) are quite variable between laboratories and dependent on the antibody and development system employed as well as the type of tissue and processing, and the desired objective (see Figure 1). Many of the individual considerations are treated in other sections of this guideline.

Procedures may be direct or indirect. In a direct method, the antibody used to detect the antigen in question is conjugated to an enzyme. After the antibody is bound to the tissue antigen, the enzyme is reacted with a chromogen/substrate to yield a color reaction. Indirect methods use a series of reagents to identify and delineate the presence of antigen. This series of reagents typically involves an unlabeled primary antibody to detect the antigen, followed by an enzyme-conjugated secondary antibody to localize the primary antibody, and the addition of the substrate/chromogen. Indirect procedures may also involve three steps, using a primary antibody, a secondary to detect the primary, and an enzyme-conjugated tertiary antibody to localize the secondary. Alternatively, another system uses a primary antibody followed by an avidin-conjugated secondary, and a biotinylated enzyme which would bind to the avidin on the secondary antibody. Primary antibodies may also be followed by biotinylated secondary antibodies, which are then detected using an enzyme-avidin or enzyme-strepatavidin complex.[4] Indirect or "bridge" methods may enhance staining intensity but may also increase background. Specific variations on and description of the individual methods are further considered in Section 7.2.

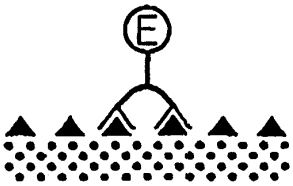

Figure 1a. Direct immunoenzyme staining. Enzyme-conjugated anitbody (E) binds directly to antigens (triangles) in the specimen.

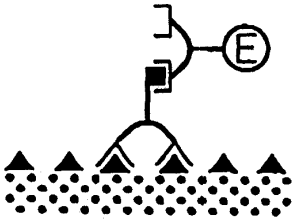

Figure 1b. Indirect immunoenzyme staining. Enzyme-conjugated second antibody (E) binds to primary antibody that is specifically bound to antigens (triangles) in the specimen.

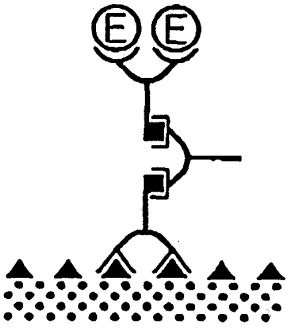

Figure 1c. Antienzyme immunostaining. A secondary antibody links primary antibody that is bound to antigens (triangles) in the specimen to antienzyme antibody that can bind molecules of enzyme (E).

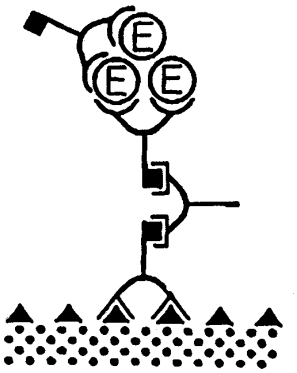

Figure 1d. Enzyme-antienzyme complex immunostaining. A secondary antibody links primary antibody that is bound to antigens (triangles) in the specimen to a complex containing molecules of enzyme (E) bound to antienzyme antibodies.

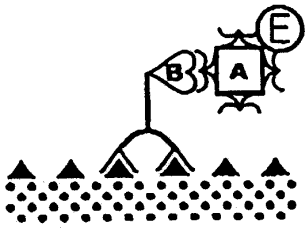

Figure 1e. Enzyme-conjugated avidin-biotin immunostaining. Enzyme (E)-conjugated avidin (A) binds to biotin (B)-conjugated antibody that is specifically bound to antigens (triangles) in the specimen.

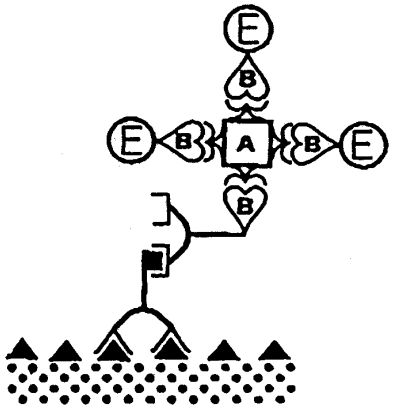

Figure 1f. ABC immunoenzyme microscopy. A biotinylated secondary antibody links primary antibody that is bound to antigens (triangles) in the specimen to an avidin (A)-biotin (B)-enzyme (E) complex.

Figure 1. Methods of Immunostaining. In each of the examples, visualization of antigen occurs when enzyme reacts with its substrate enabling deposition of chromogen to occur. Reprinted with permission from *Immunohistology in Diagnostic Pathology.* JC Jennette, ed. Copyright CRC Press, Boca Raton, FL.

7.1.1 Standard Methods

Standard methods do not necessarily follow one single protocol format, especially with regard to time and temperature. These often follow convention in a single laboratory, customized to a particular application. Modifications are made based on antibody specificities and working dilutions. Standard procedures are carried out at ambient temperature or 37 °C. Incubation times of individual steps can range from as little as 5 minutes for blocking steps, to 30 minutes, to as much as 24 to 48 hours for antibody-binding steps.

The use of a humidified incubation chamber is recommended to prevent slides from drying out, particularly when long incubation periods are employed.

A representative procedure might contain the following phases:

(1) Blocking of endogenous enzyme activity (5 to 10 minutes).
(2) Rinse or wash of slides.
(3) Nonspecific protein binding block (10 to 30 minutes).
(4) Incubation with primary antibody (30 to 60 minutes).
(5) Rinse or wash of slides.
(6) Incubation with secondary antibody (30 to 60 minutes).
(7) Rinse or wash of slides.
(8) Incubation with substrate/chromogen (<30 minutes).
(9) Rinse or wash of slides.
(10) Counterstaining (10 minutes).
(11) Rinse or wash of slides.
(12) Cover slip.

Care must be taken to optimize each of these steps. Use of too long an incubation, or too high a concentration of hydrogen peroxide, in Step 1, for example, can result in substantial loss of antigenicity, while use of too little hydrogen peroxide or too short an incubation time can result in unacceptably high background staining. These considerations are discussed in detail in the remainder of the section, particularly Section 7.4.

7.1.2 Rapid Methods

A number of methods exist which greatly reduce the times necessary for individual steps in the staining process. These methods generally do not alter the sequence of protocol steps, but rather affect the duration of one or more steps by, for example, increasing the concentration of the working reagents or altering the conditions of staining (temperature, environment). These procedures can potentially reduce incubation times tenfold or greater, to less than 30 seconds in some cases (e.g., microwave applications).

Rapid methods can have variable results with different types of antigens and/or tissues, as well as the type of processing which has been employed. However, in many cases successful staining is achieved with results comparable to standard methods, although some reports of increased background and/or lighter staining exist. Protocols which accelerate staining results may not be well suited to routine application in the laboratory or automated staining, but may prove of greatest benefit in developing a "rapid-turn-around" ("stat") staining procedure.

7.1.3 Automation

A number of considerations should be taken into account prior to implementing an automated staining system protocol within the laboratory. Initial evaluation should be done to assess the quality of the product produced (i.e., automated vs. manual) by parallel testing with the individual reagents to be placed into the automated system. This should be done on, for example, a lymphoma panel using multiple cases, to assess the variability and sensitivity between treatments and patients.

Optimize protocols according to antibody titration (see Section 7.2.3) and staining consistency, reliability, and reproducibility on the same source tissues within and between runs. Standardization of antibody titers, processing, and procedures are to be established for each tissue and sample variety (i.e., paraffin-embedded, frozen, cytologies). (For the subcommittee's rationale regarding the need for 'standardization' of various steps in the immunostaining and tissue fixation process, refer to Comment 5 in the Summary

of Proposed-Level Review Comments and Subcommittee Responses.)

Similar quality control (QC) procedures as outlined in Section 7.2.9 apply to automated methodology. In addition, instrument usage and maintenance logs should be maintained and a daily instrument start-up QC protocol established and followed according to the individual manufacturer's recommendations.

Lesser procedural requirements (e.g., for titration) may be acceptable if the instrument manufacturer's reagents are matched to their instruments, in the event the manufacturer's usage recommendations are followed. Variance from the manufacturer's protocol and/or use of a second manufacturer's reagents on the stainer necessitate more rigorous and comprehensive validation prior to implementation in a standard laboratory procedure.

Follow the manufacturer's directions for use of the instrument, routine cleaning and maintenance, and use of reagents. These factors have been determined during the design of the instrument and are important to the outcome in determining staining quality. Deviations from the manufacturer's directions require validation of the procedures by the user's laboratory. For example, if the stainer uses heat to shorten incubation times, it is possible that particular reagents are also specified. Use of temperatures or reagents other than those specified by the manufacturer require separate validation.

Depending on the instrument design for the method of reagent delivery, narrow gaps or tubing may exist where antibody or other protein-containing reagents pass or are required to remain for a period of time. While the materials used are frequently designed to minimize sticking of proteinaceous substances, it is recommended to provide a wash step using a detergent buffer to ensure thorough removal of each reagent before the addition of the next reagent. Refer to the manufacturer's recommendations for buffer composition. Generally, a Tris or PBS buffer containing a nonionic detergent such as Tween or Brij is suggested or can be obtained from the manufacturer.

Based on specific instrument design and requirements, users should determine the appropriate number of sections to be placed on one slide, where these are placed, and the maximum section thickness allowed. Verification should be made that each section on a slide is exposed to the same concentration of reagent for the same period of time. Variations of application include depositing separate aliquots of reagent on each section of a horizontal slide, flowing reagent from top to bottom over a vertically-placed slide, flowing reagent from bottom-to-top, and/or allowance for sections on opposing sides, with a narrow gap between them. The user should ensure that section thickness, number of sections per slide, and section placement on the slide are compatible with the reagent delivery system and reagent volume. These guidelines should be provided by the instrument manufacturer.

7.1.4 Inspection Requirements

Certain inspection regulations (e.g., CLIA) require that the end user validate the ICC testing or use the manufacturer's recommendations. Verification of ICC testing with a positive and negative tissue or cell control with each ICC run should be performed. If the controls do not work, the run should be discarded and no results should be reported.

The minimal regulatory performance criteria established do not exclude choosing a higher level of QC performance by laboratory. A positive and two negative controls should be used for tissue controls.

Several organizations (such as the College of American Pathologists) and regulatory agencies (such as the United States Health Care and Finance Administration) have prepared checklists which may be useful in organizing quality assurance efforts.

7.2 Generally Applicable Considerations

7.2.1 Interchangeability of Equipment and Components

Instruments, equipment and/or reagents from different manufacturers can be used interchangeably if the manufacturers have recommendations for the use or if the

laboratory can assume the responsibility for establishing the use of these components.

7.2.2 Pipetting

NCCLS document GP2—*Clinical Laboratory Technical Procedure Manuals*, recommends stating the degree of accuracy required for measuring reagents.

7.2.3 Titration of the Primary Antibody

Determination of the optimal concentration of ICC reagents is the responsibility of the laboratory. Manufacturer's recommendations can serve as a general guideline. However, dilutions for one tissue type and means of processing may not be appropriate for another containing the same antigen.

Longer incubation times (e.g., 48 hours at 4 °C) may permit greater dilutions of primary antibodies. Incubation times and temperatures should be determined before titrating the optimal antibody dilution. Testing should be done on positive control sections of known performance.

"Checkerboard" titration should be done on *each new lot* of antisera/antibody employed in the lab, titrating the primary vs. the secondary antibodies. It should not be assumed that each lot of the same antiserum will perform as the previous. Test sections with and without antigen retrieval, as is necessary.

Titration should generally be done in twofold dilutions above and below the manufacturer's recommended dilution (e.g., 1:100, 1:200, 1:400, if the manufacturer's suggested dilution is 1:200; see Table 3). If desired, further titrations can be done around the best of the three dilutions. Optimal concentration and the need for digestion should be determined by the intensity of staining obtained, specificity of the staining, and the level of background at a given dilution.

Potential problems overcome by optimizing antibody titration include the following:

- Highly concentrated (e.g., <1:50) or undiluted antibodies may result in a prozone effect, especially in areas of high antigen density.

- Detection of densely distributed antigens may be masked by steric hindrance.

- Higher concentrations of antibodies may display otherwise subtle cross-reactivities between antigens, giving rise to nonspecific staining.

- Primary antibodies of greater dilution may give less background staining, as well as being more economical in providing longer use of certain lots of antisera/antibody.

Table 3. Recommended Dilution Scheme for Optimal Antibody Titration ("Checkerboard Titration")*

2°- 3° ↓ \ 1° →	1:100	1:200	1:400
1:50	1° 1:100 2° 1:50 3° 1:50	1° 1:200 2° 1:50 3° 1:50	1° 1:400 2° 1:50 3° 1:50
1:100	1° 1:100 2° 1:100 3° 1:100	1° 1:200 2° 1:100 3° 1:100	1° 1:400 2° 1:100 3° 1:100
1:200	1° 1:100 2° 1:200 3° 1:200	1° 1:200 2° 1:200 3° 1:200	1° 1:400 2° 1:200 3° 1:200

*1°: Primary antibody
2°: Secondary antibody (if used)
3°: Tertiary antibody (if used)

7.2.3.1 Secondary Antibody

The secondary antibody is often used in a prediluted standardized format. The end user should verify the manufacturer's recommendations for the primary and secondary antibodies used in an ICC kit.

If the secondary antibody is a stand-alone formulation, the end user should establish optimal staining of each primary antibody with the secondary antibody formulation using a checkerboard titration. Careful selection of the secondary antibody can substantially improve assay performance.

7.2.4 Time and Temperature of Incubation

Times of staining are considerably variable among laboratories and individual procedures, and should be tailored to the particular application. Manufacturers' guidelines should be consulted for customizing reagents to the desired staining method. In general, staining conditions which increase the temperature of the slide and/or reagent (e.g., 37 °C incubator, microwave oven) for restricted periods of time will lower the duration required to obtain optimal staining. When increasing staining temperature beyond ambient, caution should be observed to avoid prolonged exposure to the higher temperature that may damage the tissue morphology or tissue antigens, and/or cause excessive drying of the sections.

7.2.5 Washing Slides

Slides are washed between different steps in the staining procedure to remove excess reagents. Washing decreases nonspecific or low avidity binding. Buffered physiologic saline is the usual wash solution, although some workers and all instruments also incorporate a surfactant into the wash solution. If washing is by a "dipping" method, the wash solution should be changed frequently. If a wash bottle is used, caution should be used so that the spray does not make direct contact with the sections; the spray should make contact with a portion of the slide that does not contain tissue, and the fluid then gently wash across the tissue itself.

7.2.6 Visualization Methods

Although enzymes may be used to directly label ICC primary antibody reagents, indirect labeling techniques are more frequently used, because these are usually more sensitive and do not require a separate enzyme label for each primary antibody. Direct and indirect enzyme labeling may use peroxidase, alkaline phosphatase, or glucose oxidase, but peroxidase is the one most commonly used.

Indirect conjugate-labeled antibody techniques use different formats, depending on the degree of sensitivity needed for the assay balanced with the need for simplicity in the number of procedural steps. Generally, the primary antibody-antigen reaction is amplified by application of secondary antibodies from

an animal species different from the source of the primary antibody. This secondary antibody increases the potential number of sites reacting with labeling molecule, e.g., the indirect (sandwich) procedure, unlabeled antibody methods (the enzyme bridge technique), or the enzyme-antienzyme complex method (e.g., peroxidase-antiperoxidase). Indirect enzyme labeling is a simple way to use a single enzyme-labeled secondary antibody reagent against multiple, different primary antibodies and avoid the necessity of direct labeling of many different primary antibodies.

One of the most commonly used enzyme labels is peroxidase; another is alkaline-phosphatase. Enzymes are often used as both bridging and visualization reagents, as in the peroxidase-antiperoxidase system (PAP) and the alkaline phosphatase-antialkaline phosphatase stem (APAAP). Other bridging (linking) systems include biotin-avidin, avidin-biotin complex (ABC), and protein A methods. Streptavidin is often substituted for avidin in those methods relying upon biotinylation for linking.

Enzyme-antienzyme techniques employ a primary antibody, linking or secondary antibody, and a soluble enzyme-antienzyme complex. Three different antibodies are involved in this technique. The primary antibody and the antienzyme antibody must be from identical or closely related species. The primary antibody binds to the antigen in the specimen. The secondary antibody binds to both the primary antibody and to antibodies directed against the enzyme, holding the enzyme at the site of localization of the primary antibody. The antienzyme antibodies are typically complexed to the enzyme itself, resulting in formation of large complexes and signal amplification. The secondary and antienzyme antibodies thus form a link or "bridge" between the primary antibody and the enzyme (see Figure 1). In order for the secondary antibody to form an effective bridge, the primary antibody and enzyme-antienzyme complex should be from the same species source.

Peroxidase-antiperoxidase (PAP): Endogenous peroxidase activity is usually blocked in the test specimen, which is then reacted with the primary antibody. The secondary antibody is added, followed by a complex of antibodies bound to peroxidase, and subsequently the substrate/chromogen.

Alkaline phosphatase-antialkaline phosphatase (APAAP): The test specimen is reacted with the primary antibody. The secondary antibody is added, followed by a complex of antibodies bound to alkaline phosphatase, and subsequently to the substrate/chromogen.

Protein A: Staphylococcal Protein A may alternatively be used as a bridge because of its inherent ability to bind most molecules of immunoglobulin G (IgG). The test specimen is reacted with the primary antibody. The Protein A bridge is added, followed by a complex of antibodies to Protein A bound to the appropriate enzymes, and subsequently the substrate/chromogen.

Avidin-Biotin Complex (ABC): The molecules avidin and biotin may be used as bridges between antibodies and enzymes. The molecule avidin has four high-affinity binding sites for biotin. The resulting bond is of very high avidity. This method uses a primary antibody conjugated to avidin or a secondary antibody conjugated to avidin and bound to the primary, followed by a biotinylated enzyme molecule which will bind to the avidin-conjugated enzyme, and subsequently react with the substrate/chromogen.

7.2.7 Chromogens

The enzyme label, attached directly to the primary antibody ICC reagent or indirectly on a secondary antibody, converts a chromogen substrate into an insoluble, colored end-product. The chromogen marks the site of the primary antibody-antigen reaction for study by microscopy. The choice of a particular chromogen is determined by which enzyme label is used and the color of the end product that is desired for study of the target tissues or cells.

For example, when using alkaline phosphatase enzyme label, one can use as a substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT) to form a blue-purple end product or fast red/naphthol AS-TR phosphate to form a red end product. If

peroxidase is used as the enzyme label, a brown end product is obtained with the chromogen 3,3'-Diaminobenzidine (DAB) and a red end product with the chromogen 3-amino-9-ethycarbazole (AEC). Caution should be taken in establishing compatibility between the chromogen end product and the mounting media chosen, e.g., AEC is not compatible with standard xylene-based mountants.

The simultaneous use of multiple enzyme-substrate combinations allows for multiple labeling of different antigens in the same slide preparation. These multiple-labeling techniques are technically complex, however, and seldom employed in routine clinical use.

7.2.8 Counterstaining

The ideal counterstain for an immunocytochemical preparation should contrast sharply with the chromogen and not interfere with the perception of small deposits of reaction product ("weak" reactions), while allowing clear morphologic evaluation of cellular and subcellular structure. This ideal can be approximated by selecting a counterstain whose color contrasts with that of the chromogen. Thus, for chromogens developing brown (DAB: 3,3'-diaminobenzidine) or red (AEC: 3-amino-9-ethylcarbazole) reaction products, blue or green counterstains (e.g., methylene blue, methyl green) provide optimal contrast. While a weak hematoxylin counterstain for DAB or AEC yields acceptable results, the spectral proximity of its active dye form (hematein; absorption maximum: 445 nm) to the reaction product of these chromogens reduces contrast and can actually mask weak immunocytochemical reactions. Methylene blue and methyl green (absorption maxima: 663 to 667 and 630 to 634 nm, respectively), however, allow the eye—and the camera—to distinguish the weakest DAB/AEC reaction or background stain because of their spectral distance, a physical property that can be both demonstrated and exploited by the use of blue or green filters in taking black and white photomicrographs: the filters attenuate the counterstain while enhancing the brown or red precipitate, a separation not possible in hematoxylin-counterstained preparations for which full-color photomicrography is usually necessary to illustrate results effectively.

The choice of counterstain depends not only on the choice of chromogen and the use to which the immunocytochemical preparations will be placed, but also on the mounting medium. For example, methyl green is water-soluble and of no use with aqueous mounting media.

7.2.9 Controls

In addition to external positive control sections, the interpretive process should also include a careful examination of internal positive-control cells or tissues. Although not every tested antigen has a normal internal counterpart that can be used for this purpose, many do have such determinants. As internal positive controls are processed along with the target tissue to be evaluated in the same section, they provide ideal indicators that the immunocytochemical system in place is performing satisfactorily.

Table 4 does not purport to be exhaustive in listing all appropriate antigens, but rather illustrates this principle by example.

Table 4. Paraffin Sections: Selected Internal Positive Controls

ANTIGEN	CELL OR STRUCTURE
Keratins	Normal entrapped or contiguous epithelium
S100	Peripheral nerve; dendritic reticulum cells
Smooth muscle actin	Blood vessel musculature
Desmin	Blood vessel musculature
Polypeptide hormones Synaptophysin Chromogranin	Adjacent normal tissue, e.g., normal islets in pancreatic margin or an insulinoma
GFAP	Astrocytes of adjacent non-neoplastic tumor
Hematopoietic Antigen CD1	Langerhans' cells of skin
CD1a	Langerhans' cells of the skin or thymic lymphocytes
CD3	Lymphocytes of paracortex
CD15	Polymorphonuclear leukocytes
CD20	Lymphocytes of cortical follicles
CD43	Paracortical lymphocytes
CD45RB	Lymphocytes
CD45RO	Paracortical lymphocytes
CD64	Sinus macrophages
CD68	Follicular dendritic cells and sinus macrophages
CD74	Lymphocytes of cortical follicles
CD75	Follicular germinal center cells
CD79a	Lymphocytes of cortical follicles
bcl-2	Paracortical lymphocytes (T cells)
Muramidase	Sinus macrophages
Myeloperoxidase	Polymorphonuclear leukocytes

For some antigens it is difficult, if not impossible, to find internal normal control cells or tissues (e.g., CD 30 for Reed-Sternberg cells, ALK 1 for large-cell anaplastic lymphomas.) External pathologic tissue controls should be used in these cases.

7.2.9.1 Use of Vimentin Reactivity as a Processing Quality Control Indicator

Vimentin is sometimes used as a processing quality control indicator, as are other antigens and normal tissues that may be present within a pathological specimen.

Proper processing should show uniform distribution of vimentin reactivity in tissue vessels and stromal cells. Both the intensity of staining and the distribution of staining within a field or tissue should be evaluated. Whenever possible, evaluate the tumor tissue in the context of any normal tissue that is contiguous with the biopsy specimen (the margins). Vimentin staining may be done on parallel, serial sections to those being used in the final diagnosis.

Uniformity of vimentin staining can be employed to assess the presence of fixation damage or inadequacy in the fields to be analyzed for other immunocytochemical staining. Where heterogeneous vimentin staining is evident, only those fields demonstrating the best level of intensity and distribution of the antigen are best for use in the final analysis.

Sections may be treated with protease or a similar agent prior to vimentin staining (the level of protease pretreatment required is usually commensurate with the length of fixation to which the tissue was exposed). Those specimens devoid of vimentin staining may be further treated with protease to attempt to reveal masked antigen.

7.2.9.1.1 Limitations

The suitability of vimentin staining as quality control for processing can be dependent on the epitope(s) recognized by the monoclonal (polyclonal) antibody chosen for this application. Individual antivimentin reagents should be evaluated for optimal performance in sensitivity to various anticipated processing problems. This application is primarily used to evaluate the suitability of detection of antigens potentially affected in a similar manner as vimentin. Although this would encompass most currently evaluated immunocytochemical antigens, some may have variable susceptibility to harsh processing conditions, for which intact vimentin antigenicity may not be a good indicator.

In general, those antigens with immunoreactivity in parallel with that demonstrated by vimentin staining include, but are not limited to, the cytokeratins, desmin, leuko-cyte common antigen (LCA), tissue CD markers, S100, Factor VIII, collagen type IV, estrogen receptor, progesterone receptor, c-erbB-2, epithelial membrane antigen (EMA), and carcinoembryonic antigen (CEA).

7.2.9.2 Positive Tissue Control

Positive tissue controls should be fresh autopsy/biopsy/surgical specimens that have been fixed, processed, and embedded as soon as possible in the same manner as the patient sample(s). Positive tissue controls are indicative of correctly prepared tissues and proper staining techniques. One positive tissue control for each set of test conditions should be included in each staining run. The ideal positive control contains a spectrum of weak to strongly positive reactivity; if tissue with a range of reactivity cannot be obtained, it is better that weakly reactive tissue be used as the control.

Histologic slides which have been stored with the cut surfaces exposed for prolonged periods may exhibit diminished reactivity in certain immunocytochemical assays. A decrease in reactivity may be slowed in some cases by covering the cut surface with fresh paraffin. If stored slides are to be used as positive tissue controls, the laboratory must take care to assure that they are used before reactivity to the antigen of interest has been lost.

7.2.9.3 Negative Tissue Control

Use a negative tissue control (known to be negative) fixed, processed, and embedded in a manner identical to the patient sample(s) with each staining run to verify the specific demonstration of the target antigen as labeled by the primary antibody and to provide an indication of specific background staining. **Alternatively, the variety of different cell types present in most tissue sections may serve as internal negative control sites (this should be verified by the user).**

If specific staining occurs in the negative tissue control, results with the patient specimens should be considered invalid. The

absence of specific staining in the negative tissue control confirms the lack of antibody cross-reactivity to cells/cellular components.

7.2.9.4 Nonspecific Negative Reagent Control

Use a nonspecific negative reagent control in place of the primary antibody with a section of each patient specimen to evaluate nonspecific staining and allow better interpretation of specific staining at the antigen site. Ideally, a negative reagent control contains the same isotype as the primary antibody, in the same way as the primary antibody but exhibits no specific reactivity with human tissues in the same matrix/solution as the primary antibody. Dilute the negative control antibody to the same immunoglobulin or protein concentration as the diluted primary antibody using the identical diluent. If fetal calf serum is retained in the neat primary antibody after processing, fetal calf serum at a protein concentration equivalent to the diluted primary antibody in the same diluent is also suitable for use. Diluent alone may be used as a less desirable alternative to the previously described negative reagent controls. The incubation period for the negative reagent control should correspond to that of the primary antibody.

If the primary antibody is polyclonal, use a negative reagent control in place of the primary antibody with a section of each patient specimen to evaluate nonspecific staining and allow better interpretation of specific staining at the antigen site. To prepare a negative reagent control, dilute an immunoglobulin fraction or whole serum of normal/nonimmune serum of the same animal source and adjust the protein concentration to the same as that of the diluted primary antibody, using the identical diluent.

7.2.9.5 Internal Controls Within Patient Specimens

It is very important to control for differences between the patient tissue/cells and the control slides. Every ICC evaluation of patient tissues should include an evaluation of the immunoreactivity of normal cells and tissues within the patient sample, e.g., mesenchymal cells or tissues, hematologic cells, and normal cells or tissues located in the margin or inside the sample.

When panels of several antibodies are used on serial sections, the negatively staining areas of one slide may serve as a negative/nonspecific binding background control for other antibodies. To differentiate endogenous enzyme activity or nonspecific binding of enzymes from specific immunoreactivity, additional patient tissues may be stained exclusively with substrate-chromogen, or enzyme complexes (PAP, avidin-biotin, streptavidin) and substrate-chromogen, respectively.

Nonspecific staining, if present, usually has a diffuse appearance. Sporadic staining of connective tissue may also be observed in sections from excessively formalin-fixed tissues. Use intact cells for interpretation of staining results. Necrotic or degenerated cells and calcifications often stain nonspecifically.

Table 5. Controls

Control	Target Material	Reaction Tested	Reagent(s) Tested
Vimentin Control	Vimentin in patient mesenchymal tissue	Fixation	Vimentin immunoreactivity lost with overfixation with formalin
Positive Tissue Control	Tissue or cells containing antigens to be detected	Specific antigen-antibody reaction of 1° antibody with target antigen Tests for reactivity of the 1° antibody	1° antibody
Negative Tissue Control	Tissues or cells that do not contain the antigen to be detected	Tests for cross-reactivity of 1° antibody	1° antibody
Nonspecific Reagent Control	Same tissue as tested with the 1° antibody	Assessment of nonspecific background in the patient samples, positive and negative controls, and vimentin controls	Binding of test reagents nonspecifically to the test tissues/cells
Internal Control of Patient Specimen	Serial section of patient specimen	Fixation, processing, and/or storage effects that might differ from control slides	1° antibody

7.3 Microwave Immunostaining

For microwave immunostaining it is necessary to deparaffinize and hydrate sections to water. Perform antigen retrieval, if necessary, by conventional methods. If using peroxidase in staining, also quench endogenous peroxidase by conventional incubation at room temperature using 0.3% to 0.6% hydrogen peroxide.

Most steps can use identical microwave timing and conditions, dependent on the make and model of microwave oven employed. It is important to rinse with phosphate buffered saline (PBS), wiping off excess between each step. Individual steps may require a combination of microwave and room-temperature incubations for optimal results (e.g., seven seconds microwave at high power, followed by five minutes incubation at room temperature). In general, incubation of any single step in the microwave should not exceed 40 seconds. Higher power settings may require as little as seven seconds to achieve satisfactory staining. Slides should be removed from the microwave for room temperature phases. Sufficient water load or an appropriate microwave-compatible humid chamber should be used for all microwave incubations.

Protein blocking reagents do not require room temperature incubation following microwave incubation of at least ten seconds. Excess reagent should be removed, but do not wash slides prior to addition of the primary antibody.

Counterstains (e.g., Mayer's Hematoxylin) generally require much less microwave incubation (e.g., three seconds at high power). Times greater than three seconds may lead to overstaining of the counterstain.

7.4 Troubleshooting: Common Causes of Staining Problems

The following are staining problems and common causes for their occurrence.

- No staining of any slides:
  - — Reagents omitted/not used in proper order
  - — Section allowed to dry out
  - — Sodium azide in buffer baths
  - — Too many/too few drops of hydrogen peroxide (for peroxidase procedures)
  - — Degraded hydrogen peroxide
- Control slides stain, but there is no staining of specimens:
  - — Inadequate fixation
  - — Tissue processing too harsh
  - — Antigen masked or absent
- Weak staining of all slides:
  - — Sections retain too much buffer after washing
  - — Substrate mixture more than three hours old
  - — Too much/too little substrate or substrate hydrogen peroxide
  - — Slides not incubated long enough in antibody, peroxidase reagent, or substrate mixture
  - — Buffer pH incorrect
  - — Degraded hydrogen peroxide
  - — Incorrect dilution of primary antibody, secondary antibody, or labeling system
- Control slides stain well, but specimens stain weakly:
  - — Antigen partly destroyed or present in low concentration
- Excessive background on all slides:
  - — Slides not incubated with hydrogen peroxide
  - — Chromogen deposits due to high titer of primary or secondary antibodies.
  - — Slides not properly rinsed
  - — Substrate reaction too fast
  - — Binding of avidin reagent to cellular endogenous biotin (e.g., liver, kidney, spleen, pancreas, mammary gland, fat tissue, lung)
  - — Background cellular staining in mast cells or neutrophils
- Control slides give no background or weak staining, while specimen back-ground stains too dark:
  - — Specimens contain material causing nonspecific binding
  - — Specimens contain high endogenous peroxidase activity
  - — Section thickness too large due to poor preparation or sectioning
- Control slide failure:
  - — Inappropriate control
  - — Wrong (no) reagent applied
  - — Paraffin incompletely removed

8 Validation of New or Modified Immunocytochemical/Cytochemical Assays

The numerous largely uncontrollable variables affecting the results of immunocytochemical assays, particularly when performed on routinely processed tissues, require the adoption of rigorous guidelines governing the incorporation of a new antibody or detection system into the routine test repertoire of the laboratory. The performance characteristics of an antibody or detection system should be validated and understood as thoroughly as possible in each laboratory before it can be implemented for routine use in a diagnostic context. Albert Coon's statement that "...as a tool immunofluorescence is simple in concept, but its successful use requires knowledge of immunology and skill in its application"[5] is just as applicable to immunocytochemistry today as it was to immunofluorescence in 1964.

A systematic approach to the use of a previously untried monoclonal/polyclonal antibody for the immunocytochemical localization of an antigen is the focus of this section. Since the evaluation of the primary antibody in this context requires a detection system whose performance characteristics have previously been established in the laboratory, guidelines for the latter will be addressed.

Primary antibodies to be evaluated may be monoclonal or polyclonal, obtained from commercial sources or from a research laboratory, and may be well or poorly characterized. In addition, polyclonal antibodies may be obtained as antiserum, purified immunoglobulin fraction, or affinity-purified immunoglobulin; monoclonal antibodies may consist of crude or purified fractions of ascites or supernatant fluids. The properties of each of these preparations directly influence the performance characteristics of the primary reagent.

8.1 Principle

The basis for the immunocytochemical localization of an antigen in a tissue section/cell preparation is antigen-antibody interaction, (i.e., the specific recognition of—and binding to—epitope(s) on an antigen by antibody binding site(s) [paratope(s)]). Various molecular forces (e.g., electrostatic, hydrogen-bonding, hydrophobic, Van der Waals) mediate this reversible bond, whose strength is largely determined by the spatial complementarity of the paratope (antibody) and epitope (antigen), referred to as affinity—a measurable parameter.[6] These factors influence the specificity and sensitivity of an antibody preparation which, in turn, determine its usefulness as an immunocytochemical reagent.

8.2 Preliminary Considerations

8.2.1 Antibody Specificity in Immunocytochemical Localizations

In the context of Anatomic Pathology, the practical use of an antibody to localize an antigen in a tissue section/cell preparation necessitates the ability to differentiate two levels of specificity—molecular and diagnostic. The former refers to the ability of the antibody to combine with an epitope that is a specific determinant for an antigen (e.g., the ability of antibody CAM 5.2 to specifically identify cytokeratins 8 and 18) and is governed by fundamental laws of immunochemistry and molecular biophysics. Diagnostic specificity, on the other hand, refers to the discriminative reliability of the information provided by such an antigen-antibody reaction when applied to the solution of a diagnostic problem (e.g., the ability of antibody CAM 5.2 to differentiate an adenocarcinoma from a squamous cell carcinoma). Whereas molecular specificity is a measurable, fundamental, inherent property of an antibody primarily reflecting its molecular structure, diagnostic specificity is an assigned property reflecting evolving qualitative, empirical observations on the behavior of the antibody in a given application. A basic understanding of the preceding, and especially of the fact that the specificity of antigen recognition by antibody is not absolute, is essential to the appropriate, safe, and effective application of immunocytochemistry to anatomic pathology.

8.2.1.1 Molecular Specificity of Antibodies: Cross-Reactivity

Since epitopes, particularly smaller continuous or sequential (as compared to discontinuous or conformational) ones, are not necessarily unique to an antigen, it follows that an antibody, including a monoclonal antibody preparation, can bind to different antigens sharing identical epitopes. The recognition of an antigen by an antibody is not proof of molecular identity.[7] In addition, an antibody can bind, albeit with lower affinity, to an epitope having only partial spatial complementarity with its paratope, i.e., it can recognize other antigens with similar epitopes.[7] In practice, such binding presents more of a problem with monoclonal antibody preparations than with polyclonal ones; in the latter, the low-affinity (nonspecific) antibodies are usually present in lower concentrations and their potential for cross-reactivity may be "diluted out," while the absolute structural homogeneity of monoclonal antibodies locks in their affinity and cross-reactivity.

8.2.1.2 Monoclonal vs. Polyclonal Antibodies: Affinity and Avidity

A polyclonal antibody may have a greater functional affinity than a monoclonal antibody to the same antigen, because the recognition of multiple epitopes results in overall attachment that is stronger than expected from the collective affinities of the individual antibodies.[6,8] Thus, the identical structure of monoclonal antibodies—while responsible for their exquisite specificity—precludes polyvalence and limits their binding strength to their affinity. This is not a problem with high-affinity monoclonal antibodies (in effect, the selection of such antibodies is favored in the screening of new clones) but should be considered when testing a new antibody.

Monoclonal antibodies from different but related clones can be mixed and profit from the affinity-multiplying effect of multivalence. Such antibody "cocktails" (e.g., AE1/AE3 for cytokeratins and 2B11 + PD7/26 for leukocyte common antigen, CD45) combine the best qualities of monoclonal and polyclonal antibodies and are ideal reagents for immunocytochemistry.

8.2.1.3 Diagnostic Specificity of Antibodies in Immunocytochemistry

A growing body of literature, including several texts,[9-11] and the daily practice of pathology support the fact that antibodies can be assigned degrees of diagnostic specificity. Variables precluding absolute immunologic molecular specificity, briefly discussed above, compounded by the presence on a tissue section of countless known and unknown antigens, variably altered by fixation and processing, have not prevented progress in diagnostic immunocytochemistry. However, a thorough appreciation of the technical capabilities and limitations of the discipline is essential for the deduction of valid and useful histopathologic correlations from immunocytochemical observations, especially when confronted with a new antibody.

The interpreting pathologist should also be capable of striking a proper balance between the significant subjective component of a histopathologic diagnosis and the objective result of a well-controlled, well-conducted immunocytochemical procedure. While keeping in mind that "...application of these molecular tools cannot be successful unless it is backed by a thorough understanding of the principles of histopathologic diagnosis that have been painstakingly established by generations of anatomic pathologists,"[12] the possible overriding validity of a sound immunocytochemical result should be recognized and accepted.[13-15]

8.3 Immunocytochemical Evaluation of a New Primary Antibody

A determination of optimal conditions for the localization in tissue sections of the antigen recognized by a new antibody should precede its evaluation in a diagnostic application. All available information on a new antibody should be studied carefully and taken into consideration in the design of an appropriate and logical protocol. The package insert may suffice in the case of well-characterized antibodies from commercial sources, approximating the format proposed by the Immunohistochemistry Steering Committee of the Biological Stain Commission,[16] or required by guidance documents promulgated by government agencies, such as the Food and

Drug Administration, Center for Devices and Radiological Health. Alternatively, a reference laboratory may be called upon to determine the immunocytochemical utility of a newly developed, partially characterized antibody. Most antibodies will fall between these two extremes.

8.3.1 Primary Antibody Preparation

8.3.1.1 Monoclonal Antibodies

The immunochemical characteristics of the antibody, as well the physical composition of the reagent as obtained, should be noted, as follows:

- Animal Species and Immunoglobulin Isotype

  Most monoclonal antibodies are murine, but some are produced by fusion with rat myeloma cell lines (e.g., H222 to estrogen receptor protein) which impacts on the choice of secondary reagents, as does the immunoglobulin isotype. Knowledge of the latter is also necessary if irrelevant isotype-matched immuno-globulins are used for negative controls.

- Clone Designation and Lot Number

  Various clones are available for some popular antigens (e.g., cytokeratins, CEA, p53, etc.) and many are licensed on a nonexclusive basis, so the same clone may be available from different vendors. However, only identical lot number preparations of a clone from the same source should be considered inter-changeable, since different growth conditions for the same clone can influence the reagent's performance (see below).

- Supernatant vs. Ascites

  Monoclonal antibodies are usually provided as crude or variably purified immunoglobulin fractions of tissue culture supernatant, or as ascites fluid drawn from hybridomas implanted in mice. Ascites fluid is an inexpensive way to obtain large amounts of antibody but represents a major leap backward in specificity. A pure hybridoma clone is implanted in the peritoneal cavity of a mouse to produce ascites with an usually high, but variable, titer antibody that is, in effect, polyclonal due to the contaminating immunoglobulins of the host. Potential interference by the latter should be entertained, as in the evaluation of a polyclonal antibody.

  Antibody preparations from supernatant are truly monoclonal only if the clone is grown in serum-free culture medium.[17] If grown in standard complete medium, they contain contaminating bovine (fetal calf serum) immunoglobulin. In either case, the performance of these preparations is more easily predicted, controlled, and quantified.

- Antibody Concentration

  Knowledge of the specific antibody content (in µg or ng/mL) of a reagent greatly facilitates titration for immuno-cytochemistry analysis but is often not included in the package insert. Sometimes the manufacturer may be able to provide this information upon request. The total murine immunoglobulin concentration in a reagent prepared from a murine mono-clonal antibody supernatant can, for all practical purposes, be equated with specific antibody concentration. Total protein concentration of a supernatant is useful if the nonantibody protein fraction (usually from fetal calf serum) is known. However, in an ascites-derived reagent, the great variability in hybridoma/host contributions to ascites fluid[18] minimizes the usefulness of total immunoglobulin and protein concentrations.

- Immunochemical Specificity

  The specificity of a monoclonal antibody is most commonly demonstrated by a single band identifying the corresponding antigen on a Western blot of a crude antigen preparation run on SDS-PAGE. A monoclonal antibody may also produce multiple bands if antigen molecules have been fragmented, or the antigen exists as two or more molecular species containing the same epitope but differing in molecular weight. If SDS denatures the

epitope in question, nondenaturing gels or another method should be used to demonstrate antibody specificity.[19]

Available information on the nature of the epitope from epitope mapping and sequencing studies[6] aid in anticipating possible cross-reactivities (see Sections 7.2.9.3 and 8.2.1.1), effects of fixation, enzyme digestion, and antigen retrieval methods, etc.

- Affinity/Avidity

The dissociation constant (K_d) of a monoclonal antibody and the avidity constant (K_{avid}) of monoclonal antibody "cocktails" (see Section 8.2.1.2), measuring the strength and reversibility of the antibody-antigen bond,[6] are important indicators of the probability of success of immunocytochemical localization. Antibodies with K_d/K_{avid} 10^{-8} are likely to produce a strong reaction, while those with values 10^{-6} are generally weak reactants that may also lead to undesirable cross-reactions under conditions that favor their low-affinity binding. Dissociation and affinity constants may thus (when available) assist substantially in reagent selection and optimization. However, these are frequently not available from commercial sources.

8.3.1.2 Polyclonal Antibodies

Although basic immunochemical principles apply as with monoclonal antibody preparations, some salient differences should be noted as follows:

- Animal Species

The animal species is of importance with respect to choice of blocking agents, secondary reagents, and the use of irrelevant immunoglobulins for negative controls. In this context it is also important to note that there are immunoglobulin antigenic determinants shared by different species (e.g., between sheep and goats).

- Lot Number

The numerous variables influencing *in vivo* antibody production can effect vast differences in antibody content from bleed to bleed, so lot numbers acquire even greater importance with polyclonal antibodies.

- Antibody Concentration

Since the immunoglobulin fraction of an antiserum consists of a heterogeneous mixture of antibodies with a wide spectrum of specificities and affinities, its specific antibody content (in µg or ng/mL) is more difficult to obtain than that of a monoclonal antibody preparation. Various purification methods[18,19] efficiently separate the immunoglobulins from other serum proteins and yield preparations with known total immunoglobulin concentration and total protein concentration. This information can be used to set up a titration, since 10% of the immunoglobulin fraction is generally considered the upper limit of specific antibody content in hyperimmune sera.[18]

Affinity-purified antibodies,[18,19] the specific antibody fraction separated through binding to antigen, represent the most specific preparation of polyclonal antibodies available. Although theoretically ideal for immunocytochemistry, in practice affinity separations, apart from involving considerable time and expense, frequently exclude the best (highest affinity) antibodies, because they cannot be easily eluted from the antigen. Harsher elution conditions can irreversibly inactivate these antibodies,[18,19] although conditions can be modified to prevent or minimize their loss.[6] Nevertheless, probably because of the affinity-multiplying effect of multivalence, these reagents can be extremely effective.

- Immunochemical Specificity

Western blots are also useful to demonstrate specificity of polyclonal antibodies, particularly using crude antigen preparations on SDS-PAGE (see Section 8.3.1.1)

to exclude reactions with possible contaminants in the antigen preparation. Immunoelectrophoresis, rocket electrophoresis, and crossed immunoelectrophoresis[19] are other methods employed for polyclonal antibodies, their choice usually dictated by the nature of the antigen preparation.

- Affinity/Avidity

  As previously discussed, the multivalence of a polyclonal antibody preparation confers a superior functional affinity (avidity) that frequently outweighs the disadvantages of heterogeneity and assures their place in immunocytochemical procedures.

8.3.2 Initial Titration of Primary Antibody: Practical Considerations

Although twofold dilutions above and below the manufacturer's recommended dilution is a good approach to the initial titration of an antibody (see Section 7.2.3), dilution recommendations are not always available. General guidelines deduced from the type of antibody preparation (see Section 8.3.1) can then be applied, based on a *specific* antibody concentration range of 1 to 5 µg/mL for an *initial* titration. The optimal working concentration for many, if not most, antibodies in routine use in immunocytochemistry lies below 1 µg/mL, particularly with the more sensitive secondary detection systems; however, it is useful to include concentrations up to 4 to 5 µg/mL, because (1) frequently this is a presumed *specific* antibody concentration that may actually be much lower in polyclonal or ascites preparations, and (2) it is useful to have data on the immunocytochemical behavior of an antibody when used above optimal concentration.

8.3.2.1 Monoclonal Antibodies

Available information (e.g., total protein concentration, total antibody/Ig concentration, specific antibody/Ig concentration) can be used to formulate the initial titration. Dilutions for a titration of 1, 2, and 4 µg/mL are easily calculated if the specific antibody concentration is known. If only total antibody/Ig or protein concentrations are known, however, dilutions must be based on broad assumptions whose accuracy should always be investigated if results are questionable.

For ascites preparations, specific antibody represents 10 to 90% of the total antibody concentration and 5 to 50% of the total protein concentration.[18] For practical purposes it would be reasonable to assume 50% and 25% values, respectively, and thus, for example, the proposed initial titration for an ascites preparation with a total antibody concentration of 8 mg/mL is 1:1000 (50% of 8 mg/mL ÷ 4 µg/mL = 1000), 1:2000 (2 µg/mL), 1:4000 (1 µg/mL).

For supernatant preparations *from complete media* (containing 10% fetal calf serum), a reasonable assumption is that specific antibody represents about 5% of total antibody concentration, while in preparations from *serum-free media* (see Section 8.3.1.1), the specific and total antibody/Ig concentrations are essentially equal.[18] However, specific antibody/Ig concentration may represent as little as 1% (or even less) of the total protein concentration depending on the protein content of the medium and the productivity of the hybridoma in that medium.

8.3.2.2 Polyclonal Antibodies

Except for affinity-purified preparations (see Section 8.3.1.2) in which theoretically all antibodies are specific antibodies, the specific antibody/Ig component of polyclonal antibody preparations is no more than 10% of the total antibody/Ig fraction, and a somewhat lower percentage of the total protein concentration. An assumption of 5% specific antibody/Ig content is a reasonable starting point and, for example, the proposed initial titration for a polyclonal antibody preparation with a total antibody/Ig concentration of 8 mg/mL is 1:100 (5% of 8 mg/mL ÷ 4 µg/mL = 100), 1:200 (2 µg/mL), 1:400 (1 µg/mL). In nonpurified (whole) antiserum the total antibody/Ig concentration is usually about 10 mg/ml, and thus—in the absence of any information—an antiserum could be initially titrated at 1:125 (5% of 10 mg/mL ÷ 4 µg/mL = 125), 1:250 (2 µg/mL), 1:500 (1 µg/mL).

It must be emphasized that all of the initial antibody/Ig and protein concentrations

mentioned above for monoclonal and polyclonal reagents refer to undiluted preparations. In practice, however, antibody preparations are not uncommonly diluted for various reasons before reaching the end user. If no component information is provided in such cases, the use of a simple protein determination method (e.g., UV or Lowry) is highly recommended.

8.4 Properties of the Target Antigen

The localization of an antigen is facilitated by an understanding of its biochemical and physiological properties, particularly as they relate to the distribution and condition of the native antigen as found in the tissue or cell *in vivo* and in the excised specimen, before and after fixation. In the case of monoclonal antibodies that localize one epitope within an antigen, attention should be focused on the properties of that region of the antigen.

8.4.1 Biochemical Properties

Most antigens relevant to diagnostic pathology are proteins or have a significant protein component (e.g., glycoproteins, mucins, etc.), and greater specificity in their localization is usually obtained with antibodies recognizing this component (rather than a carbohydrate side chain). In the case of monoclonal antibodies, available information on the nature of the epitope from epitope mapping and sequencing studies[6] aid in anticipating possible cross-reactivities (see Section 8.2.1.1), effects of fixation, enzyme digestion and antigen retrieval methods, etc.

8.4.1.1 Epitope Characteristics

An epitope may be as small as a monosaccharide,[20] but usually consists of up to five or six amino acid residues and, in the case of a globular protein, as many as 16 amino acid side chains may be in contact with the antibody.[21] Since epitopes are not only continuous (sequential) but also discontinuous (conformational, assembled), conditions effecting not only cross-linking (e.g., aldehyde fixation) or cleavage (e.g., enzyme digestion) of amino acid residues, but also unfolding or otherwise altering the shape of the molecule (e.g., breaking disulfide bonds), may preclude antibody binding. In highly conserved polypeptides and simple proteins (e.g., pituitary hormones) this potential problem is minimal, but acquires importance with globular proteins and mucins.

8.4.1.2 Solubility and Diffusion

The solubility of the antigen and its ability to diffuse in tissue prior to fixation are important considerations in its localization. Steroid hormones, for example, are variably eluted from tissue by the organic solvents used in routine processing, and their localization in paraffin sections (or in alcohol/acetone-fixed frozen sections), although possible, is difficult to correlate with *in vivo* content, and a negative result is not reliable.

The diffusion of extracellular antigens (e.g., serum proteins) into the cell (after breakdown of membrane potential or enzymatic damage following excision), or of cellular antigens to the interstitial compartment, can produce artifactual localizations that impede accurate interpretation. Examples include the localization of both heavy and light chains in the same plasma cell and the localization of prostate-specific antigen in the connective tissue surrounding prostatic acini (that may be confused with nonspecific "background").

8.4.2 Physiological Properties

Rate of synthesis, release and intracellular accumulation, *in vivo* enzymatic cleavage, and ligand-receptor interaction are just a few of the factors that affect the localization of antigens in tissue, as exemplified by α_1-antitrypsin and human chorionic gonadotropin (hCG). α_1-Antitrypsin is produced by hepatocytes but is only localized in these cells in cases of α_1-antitrypsin deficiency and occasionally in other abnormal conditions.[22] Monoclonal antibodies have been produced which can differentiate the intact hCG molecule from its subunit; others, recognizing the receptor binding site of the free molecule, fail to detect receptor-bound hCG and are capable of blocking hormone action.[23]

8.5 Tissue

Decisions on the choice of tissue and tissue preparation methods should be guided by available information on the antigen/epitope,

as well as on the antibody, as suggested in Sections 8.1 and 8.2.

8.5.1 Tissue Preparation

Any process that alters the native state of an antigen may affect its epitopes and, in turn, their recognition by antibodies in tissue. A comprehensive discussion is beyond the scope of this document but, in the context of the initial testing of a new antibody, a brief consideration of the following is appropriate (see also Section 5).

8.5.1.1 Frozen vs. Paraffin-Embedded Sections

In the absence of data on the localization of an antigen in paraffin-embedded tissue, it is prudent to trisect the target tissue, snap freezing one part and fixing the others in 10% buffered formalin and an alcohol-based fixative (e.g., Carnoy solution). Given that formalin is the routine fixative in most anatomic pathology laboratories, and the impressive results obtained with antigen retrieval methods in formalin-fixed tissues, initial testing may be done on such routinely processed tissue. Other modes of fixation and/or frozen sections can subsequently be explored if results exclude the feasibility of this approach.

8.5.1.2 Fixation

In general, the preservation of morphologic structure is inversely related to antigenic integrity. Thus, the extensive inter- and intramolecular cross-linkages of the bifunctional aldehyde-based fixatives, while excellent for routine light and electron microscopy, interfere with antigen recognition through denaturation and masking by steric hindrance. Alcohol-based fixatives, on the other hand, immobilize by precipitation with minimal, largely reversible denaturation, but dehydrate and shrink the tissue—compromising morphologic detail. Reference to the procedure section, as well as to basic discussions on the application and mode of action of the various fixative preparations in use in immunocytochemistry,[9,24,25] will assist in choosing the appropriate fixative for a new antigen-antibody combination.

8.5.1.3 Antigen Retrieval Methods

Procedures primarily involving the heating of tissues have been described which largely reverse the adverse effects of fixation on antigenicity. They represent an extremely useful development[26] particularly since most localizations in a diagnostic setting are retrospective and the choice of fixative is not an option. Their variable effect on diverse antigens,[26,27] plus an incomplete understanding of their mode of action, however, suggest a careful, systematic approach to their application (see Section 6.1).[28]

8.5.1.4 Enzymatic Digestion

The use of trypsin, pepsin, pronase, etc., to restore antigenicity has fortunately been made largely obsolete by heat-based antigen retrieval methods. However, mild enzymatic digestion in combination with heat-based antigen retrieval enhances the localization of some antigens (e.g., cytokeratins, type IV collagen)[26] and can be useful in special situations.

8.5.2 Tissue Selection

Performance testing undertaken when first implementing a new assay within the laboratory should generally include:

- Normal tissue testing (for differentiation antigens)
- Diseased-tissue (usually tumor) sensitivity and specificity testing (for differentiation or disease-related antigens)
- Prognostic value verification (when appropriate).

8.5.2.1 Normal Tissue Testing

Normal tissue testing is performed to gain information on antigen specificity and staining patterns. Although normal tissue testing does not replace testing of tumor tissue, it may provide useful insights in routine clinical use, as well as a perspective on the range of background staining which may be seen.

8.5.2.2 Normal Tissues to be Tested

The choice of normal tissues to be tested depends upon the intended use of the test system. Assays for differentiation antigens are typically tested on tissue panels, which include those tissues most likely to be encountered during utilization of a particular assay.

8.5.2.3 General Differentiation Antigens

Normal tissues included in panels for assessing general differentiation antigens, such as cytokeratin, carcinoembryonic antigen, and prostate-specific antigen, include:

- Brain (including both cerebral and cerebellar gray and white matter)
- Skin
- Salivary gland
- Esophagus
- Stomach
- Duodenum
- Jejunum
- Ileum
- Colon
- Liver
- Pancreas
- Lung (including both bronchi and mesothelial surfaces)
- Adrenal gland
- Thyroid
- Kidney
- Breast
- Endometrium
- Cervix (squamocolumnar junction)
- Prostate
- Testes
- Heart
- Skeletal muscle
- Spleen
- Lymph Node
- Tonsil
- Thymus (if possible)
- Bone marrow.

This panel includes representative tissues from each major system in which a tumor is likely to be found, and includes representatives of most major tissue types within the body. Ideally, the tissues used for such a study should be obtained and fixed in a manner similar to that method by which most clinical specimens are obtained (typically surgical resection). However, the use of autopsy tissue is generally acceptable.

8.5.2.4 Neuroendocrine Antigens

Panels of normal tissues used for intralaboratory validation of assays for neuroendocrine markers generally include:

- Pituitary
- Parathyroid
- Thyroid
- Adrenal (cortex and medulla)
- Pancreas
- Duodenum
- Stomach
- Testes
- Lung, including bronchi
- Skin.

8.5.2.5 Central Nervous System Antigens

Panels of normal tissues used for intralaboratory validation of assays for central nervous system markers generally include:

- Cerebral cortex
- Pons
- Medulla
- Cerebellum
- Basal ganglia
- Spinal cord
- Pituitary.

8.5.2.6 Hematopoietic Antigens

Panels of normal tissues used for intralaboratory validation of assays for hematopoietic markers generally include:

- Lymph node

- Spleen
- Tonsil
- Bone marrow
- Thymus.

8.5.2.7 Number of Tissues to be Tested

Although testing against tissues derived from three to five individuals provides a greater check of consistency of reaction than does testing of tissue from a single individual, the latter is acceptable in the vast majority of cases.

8.5.2.8 Diseased-Tissue Testing

The number and types of diseased tissues to be tested depends upon the range of application intended in normal clinical use. It is generally prudent to test tumor differentiation antigens on several easily diagnosed and well-characterized examples of each type of tumor among which the assay will be used to assist in discrimination. While the wide spectrum of tumor behavior precludes development of absolutely reliable algorithmic approaches to tumor diagnosis or assay validation, it is both expensive and unreasonable to subject a reagent intended to assist in classification of malignant lymphoma to testing on a range of pancreatic endocrine neoplasms. The guidelines below, though not all-encompassing, outline the general sort of testing which seems appropriate.

8.5.2.9 Differentiation Antigens

8.5.2.9.1 General Markers for Lineage

General lineage markers require the most extensive validation, when used to differentiate among the major classes of tumor. Pan cytokeratin antibodies, reagents for cytokeratin 7, cytokeratin 20, chromogranin, vimentin, leukocyte common antigen (LCA), S100, and HMB-45 fall into this category. In general, a new reagent introduced for this purpose requires the most extensive testing.

8.5.2.9.2 Markers for Hematopoietic Differentiation

Validation of these markers requires testing only of hematopoietic neoplasms. Typical panels of tissues included in the validation are as follows:

- Follicular hyperplasia
- Follicular lymphoma
- Sinus histiocytosis
- Hodgkin's disease (lymphocyte predominance, nodular variant LP, mixed cellularity, nodular sclerosis)
- Small lymphocytic lymphoma
- Intermediate-cell lymphoma (including mantle zone lymphoma)
- MALToma
- Immunoblastic sarcoma of B cell type
- T-cell lymphoma
- Anaplastic large cell (Ki-1) lymphoma.

8.5.2.9.3 Markers for Neuroendocrine Differentiation

General markers for neuroendocrine differentiation, such as neuron-specific enolase, chromogranin A, synaptophysin, Leu-7, and bombesin should be tested against a wide variety of tumors, particularly neuroendocrine tumors and adenocarcinomas. More specific differentiation markers, such as thyrocalcitonin, need be tested against a more limited range. For example, thyrocalcitonin assays should usually be performed on several thyroid and parathyroid tumors; insulin assays on several types of hormonally characterized pancreatic islet-cell tumors; and pituitary hormone assays performed on a variety of hormonally analyzed pituitary tumors, as well as other types of tumors arising within the cranial vault.

8.5.2.9.4 Markers for Infectious Diseases

Validation of these markers requires testing only of specimens demonstrating similar morphologic changes, or representing infections closely related to that for which testing is to be performed. For example, when testing an antibody directed against herpes simplex virus, it is useful to validate it against tissues infected with Zoster or Epstein-Barr virus. Similarly, when testing an antibody directed against *Candida spp.*, it is useful to validate it against other fungal diseases.

8.5.2.9.5 Prognostic Markers

Validation of prognostic markers is difficult. When a "home brew" assay is used, the laboratory should perform a study demonstrating prognostic value in its own patient population before concluding that literature results may be generalized to the specific laboratory. The design of studies for prognostic factor validation is beyond the scope of this guideline; prudent fiscal practice suggests that prior to clinical adoption of an immunocytochemical prognostic factor, careful attention should be paid to the possible redundancy between the immunocytochemical data and readily obtained clinical or histologic data. This requires use of multivariate analysis methods, such as stepwise proportional hazards models.

8.6 Number of Cases Required

There is no hard and fast rule for determining the number of cases on which a particular assay should be validated. As a rule of thumb, it takes about 15 or 20 cases in each of two groups to determine if antibody staining is significantly more likely in one group of cases than another. For this reason, it is generally appropriate to perform a newly introduced assay on a minimum of 15 to 20 cases in which the diagnosis is well-established, and in 15 to 20 cases each of the more common tumors likely to be histologically confused with the "index type." Although a more formal evaluation is preferable, an alternative approach which is more practical for most laboratories is to implement any new assay for a trial period of one to three months before attempting to utilize the results to classify truly difficult tumors. During this period the assay can be employed on relatively straightforward cases of many types, the results summarized, and a qualitative appreciation of the assets and pitfalls of a new assay established.

It is not uncommon for previously unappreciated characteristics of a new assay to manifest themselves after the assay has been implemented into routine practice. Laboratories which avail themselves of the three to six months following introduction of a new antibody to perform a formal "postintroduction" surveillance may increase the effectiveness of their utilization of these new antibodies.

8.7 Test Kits

The use of prepackaged test kits may reduce inter- and intralaboratory heterogeneity, and speed up the process of new assay implementation. In general, care should be taken to use these reagents according to the manufacturer's instructions. Laboratorians should remain aware that differences in tissue handling prior to immunocytochemical staining may significantly affect the results obtained with these test kits, however, and must not shirk the responsibility for performing some in-house validation of test performance characteristics prior to relying upon these test kits in diagnostic decision making.

In general, manufacturers recommend not adjusting the antibody titer, but suggest that the end user control staining intensity by changing incubation time and/or temperature.

9 Reporting of Test Results

9.1 Format/Location of the Report

The immunocytochemistry results should be incorporated into the primary anatomic pathology report (surgical pathology, cytology, or autopsy) whenever possible. If necessary due to time constraints, the results of immunocytochemistry studies may be reported in an addendum to the primary report, which will be distributed to all recipients of the primary report. If an addendum is employed, the primary report should indicate that the studies are pending, and that an addendum will follow. The report (whether part of the primary report or freestanding) should include the rationale for the studies performed, and the differential diagnosis.

The immunocytochemistry report, if free-standing, should include full patient demographics and should make clear reference to the primary anatomic pathology report number if a separate numbering system is employed. If immunocytochemistry studies should be performed at a significant interval after the primary report was generated, they

should still be reported and a rationale for the belated studies given (i.e., comparison of immunophenotype of primary tumor with a recurrence, reconsideration of a diagnosis due to unexpected clinical course, etc.).

9.2 Nature of Specimen

The report should include a description of the origin of the specimen (e.g., surgical biopsy, cytology of a cavity fluid, etc.). Preservation of the specimen should be included, especially if the fixative is other than the standard buffered formalin, which may be presumed if no fixative is specified. Length of fixation time should be recorded, especially if this is known to be other than the standard of 24 hours or less. The processing and embedding technique should be specified (paraffin embedded, cryostat section, etc.).

9.3 Immunoreagents Employed

Antibodies should be listed both by clone number and by generic description (i.e., HMB 45, antimelanoma antibody). Specific details of procedure, such as antibody titer, antigen retrieval, lot number and source, etc. should be available in the laboratory procedure manual or laboratory records, but need not be included with each report. When the procedure manual is revised, prior procedures should be retained, and the dates during which they were in effect should be recorded.

9.4 Results Reporting

All antibody studies performed should be reported, including negatives. Reactivity of controls should be reported, including both internal and external. Of particular importance is the clear definition of the relationship of the concentration gradient of the epitope under investigation to the intensity scale used for result reporting.

Localization of staining should be described as appropriate as follows:

- Lesional tissue vs. normal (internal control)
- Nuclear vs. extranuclear staining, including localization within the cell (cytoplasm vs. membrane, apex vs. base, etc.)

9.4.1 Quantitation

Immunocytochemical reactions are not and should not ordinarily be considered to be stoichiometric. Tissue antigens are not always presented in a way that enables all antigens within a tissue to be equally available for antibody reaction. In addition, steric interference may impede antibody binding when antigen density is high. Thus, the utility of quantitative and semiquantitative immunocytochemical methods should not be assumed without considerable experimental verification.

Visual semiquantitation of results may be offered as appropriate in those cases where quantitation by image analysis is not performed. This may include the following:

- Estimate of percentage of cells which are positive in lesional tissue and in normal tissue
- Assessment of staining intensity, and a complete description of the controls which are used to define this intensity range.

Quantitation by image analysis may be valuable, but the limitations should be appreciated and include the following:

- The intrinsic limits of the stain itself. Image analysis cannot compensate for a poor stain, and may in fact accentuate problems of high background, etc.
- Sampling bias. Interactive image systems, where the operator selects the field to be imaged, are subject to considerable selection bias, no less so than visual assessment.
- Not all chromogens are absorbing dyes, and, therefore, absorbance data may be misleading.

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Summary of Proposed-Level Review Comments and Subcommittee Responses

MM4-P: *Quality Assurance for Immunocytochemistry; Proposed Guideline*

General Comments

1. Several comments were received that expressed concern regarding the lack of detailed guidelines on such general laboratory quality assurance issues as temperature checks in baths and freezers, reagent dating, and so forth.

- **This guideline is not intended to be used in isolation, but rather in conjunction with other NCCLS documents, which cover general laboratory quality assurance issues in great detail. The subcommittee does not believe it is appropriate to repeat these recommendations or specifications in this guideline.**

2. Several comments were received that expressed concern that the guideline does not deal with proficiency testing.

- **The subcommittee believes that the importance of laboratory participation in external quality assurance activities, such as proficiency testing programs, is a given. In this document we assume that the laboratory will take part in such programs when available. However, it is beyond the scope of this document to suggest the elements required of such external quality assurance activities. We have added a statement in Section 2 (Scope) to indicate the importance of external quality assurance activities in this and every area of the clinical laboratory.**

3. Several commentors expressed concern that the subcommittee was composed of individuals entirely from the United States.

- **The subcommittee shares this concern. All NCCLS member organizations were originally asked to submit names for consideration. The nationality of the subcommittee members reflects the extremely limited number of nominations received. However, NCCLS has recently appointed two international advisors.**

4. Several commentors expressed strong opinions regarding the appropriate use of the terms “immunocytochemistry,” “immunohistochemistry,” “immunomicroscopy,” “immunohistology,” and similar terms.

- **Many terms are in widespread use. The document has been modified to demonstrate that, for the purposes of the document, the subcommittee is using these and similar terms interchangeably.**

5. Throughout the document the need for "standardization" of various steps in the immunostaining process and in tissue fixation is emphasized. I have a problem with this term as I do not believe that "standardization" as an aim is achievable. I do not believe that it will be possible to cajole, compel, or advise every laboratory to perform an identical procedure. The results of the immunostaining procedure will be strongly dependent on the fixation, and we know that every laboratory throughout the world and even throughout the same country or town will have a slightly different fixation time, type of fixative, or time lapse between removal and immersion in fixative. For this reason, the aim should be optimization of the immunostaining procedure for the individual laboratory to suit its peculiar method of fixation of tissue. By optimization, I mean the adjustment of the immunostaining procedure to produce the best or optimal results for the tissue fixed and processed in that laboratory.

- **The subcommittee basically agrees with the sentiment behind this statement, and the document is written in a way that reflects the large variation in laboratory practices. Nevertheless, the subcommittee is also convinced that greater standardization of tissue handling and assay performance would result in a decrease in interlaboratory variability in immunocytochemical testing.**

6. Concern was expressed about the detail devoted to preparation of slides.

- **This section has been revised and shortened slightly, but the subcommittee notes that many of the difficulties encountered in the interpretation of immunocytochemical stains result from errors in slide preparation that occur prior to immunocytochemical staining.**

7. The methodology of immunostaining is allotted one page of text and one page of diagrams. Surely, this area is foundational, and it deserves a detailed comparative review.

- **The subcommittee believes that a prudent laboratorian would do well to consult one of the many excellent texts on the subject, and that a detailed discussion is beyond the scope of this guideline.**

8. Several individuals commented that the guideline would benefit from some "best methods" given in detail, and also recommended recipes for commonly used buffers, antigen retrieval solutions, etc.

- **The subcommittee understands the utility of a document that provides such best methods but does not believe it is possible at the present time to develop a consensus on what constitutes these best methods, particularly since they may depend upon tissue factors that vary significantly from laboratory to laboratory. However, some references to papers on antigen retrieval have been included. These references provide several such recipes.**

Abstract

9. Should read '...a thorough understanding of specificity, sensitivity, technical artifacts, and limitations...'

- **The subcommittee agrees with this suggestion and has reworded the document accordingly.**

Foreword

10. Several comments were received stating that *in-situ* hybridization and immunocytochemistry being carried out on the same section is unrealistic for most laboratories.

- **The subcommittee agrees and has modified this section accordingly.**

Section 1

11. Introduction should read, 'Proper interpretation and reporting of tissue biopsies....'

- **The subcommittee agrees and has reworded this sentence.**

Section 3

12. Several comments were received regarding various definitions, including those of "affinity," "antibody," "antigen," "biotin," "blocking," "diagnostic test," "enzyme conjugate," "immunocytochemical assay," and "reporter molecule."

- **The subcommittee has used the consensus definitions that appear in the most current edition of NCCLS document NRSCL8 *Terminology and Definitions for Use in NCCLS Documents*, and believes that, for the sake of uniformity in NCCLS consensus documents, it is appropriate to do so in this document. These definitions may be revised in the future.**

Section 4

13. I am surprised at the scarcity of health and safety information, e.g., potential hazards of solvents, chemicals such as diaminobenzidine, amino-ethyl-carbozole, etc.

- **The subcommittee believes that details having to do with toxicity and waste material are best taken care of by the use of *Material Safety Data Sheets*, and that laboratorians must adhere to both good laboratory practices and the various laws and regulations pertaining to the handling and disposal of chemicals used in the immunocytochemistry laboratory. These concepts have been introduced into the document.**

Section 5

14. A great deal of emphasis is placed on tissue processing, and this is very important. Are there not currently published guidelines on fixation and embedding?

- **Although there are published guidelines for the handling of cytologic specimens, there are no current NCCLS guidelines for tissue fixation and embedding.**

Section 5.2

15. The current wording is not compatible with the requirement to reduce interlaboratory variability. It should be only necessary to collect specimens into a formalin fixative and glutaraldehyde. Also, fresh frozen samples may be needed for certain types of work. No other fixative should be required unless specified for special purposes. This should be determined by local requirements. Examples such as B5, Bouin, etc. perhaps should be mentioned at this point.

- **The subcommittee is not certain of the intent of this comment. In the subcommittee s opinion, the most effective way to reduce interlaboratory variability is to increase accuracy of diagnosis in all laboratories. In our opinion, the best way to achieve this goal is to optimize sample handling in a way that permits diagnoses to be made on tissue which has been optimally handled since the time of excision. This may involve use of frozen specimens, special fixatives, and so forth, all of which are best determined by the nature of the tissue, the biological question to be answered, and the technology available at the time the biopsy is obtained.**

Section 5.2.2

16. Rapid preservation by freezing is, of course, not relevant if you do immunohistochemistry on paraffin embedded material (as may be the case with estrogen receptors).

- **The subcommittee has reworded this statement to be more specific.**

17. Some proteins, e.g., estrogen receptor, are quite labile, and it is important that the larger specimens be transferred promptly to the pathology laboratory where they should be sliced to facilitate fixation in a formalin fixative.

- **A statement on the need to transport specimens promptly to the pathology laboratory for gross examination and further processing has been added.**

Section 5.3.2

18. Microwave oven fixation is an area fraught with potential problems and must be a source of interlaboratory variation. Using heat to fix the larger, more dense specimen, e.g., breast lump, will probably mean that the outer zone will be optimally fixed. The fixation of the outer zone will then slow the penetration of the fixative to the inner parts, thus exposing the epitopes at the center to heat damage.

- **The subcommittee understands the commentor s concern, but believes it is adequately addressed by the guideline s statement that Tissue specimens should be no more than 5 mm in thickness.**

Section 5.3.2.2

19. This section talks about staining when clearly this section is about fixation.

- **The first use of the term staining was erroneous, as noted by the commentor, and has been changed to fixation.**

Section 5.3.3

20. More details about the antigen retrieval techniques of cytologic specimens are needed as they are dependent on the type of fixation. What are the immunocytological consequences of, e.g., a previous Giemsa staining? How is control material obtained?

- **We have added a statement in Section 6.1 (Antigen Retrieval) regarding the potential loss of tissue during antigen retrieval procedures on cytologic material, and noted that immunocytochemical staining can be performed over previously Pap stained material. Subcommittee members have generally avoided the temptation to stain over a previously Giemsa-stained specimen, because of concern that changes in immunoreactivity will have resulted from air-drying. Thus, the subcommittee s experience in this area is very limited.**

  Obtaining cytologic controls is always a very difficult problem, with solutions that must be customized to the individual medical institution.

Section 5.3.3.1

21. States 'long-term storage may not be acceptable for ICC.' We have retrieved routine diagnostic antigens in tissues fixed in formalin for up to two years, but do accept that fixation over many months can be detrimental to the processing qualities.

- **The subcommittee does not perceive any difference between the observations in this comment and the language of the proposed guideline. It is sometimes possible to successfully perform immunocytochemical assays on tissue that has been fixed in formalin for prolonged periods, but this depends greatly on the conditions of fixation and the antigen to be assayed.**

22. I do not agree with statement 'NBF is not the fixative of choice for ICC procedures.' The vast majority of diagnostic immunocytochemistry in the world is carried out on formalin-fixed tissues with many employing the NBF variety. Furthermore, a paper by N.A. Byron entitled "Antigen retrieval on paraffin-embedded tissue fixed with various fixatives*, J Cell Path.* 1997;2,2:53-66, provides some evidence that NBF enables good labeling of antigens in conjunction with appropriate antigen retrieval techniques.

- **The language is not the fixative of choice has been changed to may not be the fixative of choice, although most subcommittee members believe zinc formalin to be a better alternative**

than neutral buffered formalin.

23. Fixation times of 24 to 48 hours necessary for complete fixation are unattainable in most hospitals, and impossible to control. Most often, the decision to perform immunological procedures is made AFTER the pathologist has received and viewed the H&E. At that point, there is no way to control the time or type of fixation that the tissue received. It would also be impossible to document after the fact the amount of fixation that each specimen had received. If it cannot be documented, then QC and QA cannot be performed. State that the tissue must be completely fixed, and that fixation time should be standardized.

- **The subcommittee believes that the time required for complete fixation is determined by the tissue, not the local circumstances. The statement in question is intended to be informative, and may be useful for a pathologist who is having difficulty in obtaining the control necessary to assure optimal diagnosis in his/her institution.**

Section 5.4

24. Several commentors noted that the subject of decalcification did not appear in the document.

- **A section on decalcification (5.4.3) has been added.**

Section 5.5.1

25. Several commentors indicated that it would be worthwhile to cross-reference Section 6.2 at several other places within Section 5.5.1.

- **A cross-reference to Section 6.2 has been added.**

26. We recommend adding warning statements regarding the use of toluene and chloroform, which are hazardous and flammable materials.

- **The section on safety (Section 4) was expanded to handle these and other concerns.**

Section 5.5.1.1

27. The first sentence is very confusing. "Dehydration and clearing are necessary to remove the water within the tissue, and subsequently clear the dehydration agent from the tissue; both in preparation of embedding in paraffin." It reads that dehydration and clearing both remove water, and that both dehydration and clearing will clear the dehydration agent from the tissue. The portion after the semicolon has no verb. Rewrite the sentence to read: "Dehydration removes the water from within the tissue and cells. Clearing agents subsequently remove the dehydration agent from the tissue. Adequate dehydration and clearing are both necessary in the preparation of tissue for paraffin embedding."

- **The subcommittee has adopted the proposed language.**

Section 5.5.1.3

28. Several commentors suggested that thinner sections are sometimes appropriate.

- **The subcommittee agrees, and the statement has been reworded to state that sections should be cut at 6 μm or less.**

29. Several commentors suggested that hot plates not be used to dry slides, since they can "be subject to wide swings in temperature." Several different temperatures and methods of drying slides were mentioned.

- **We believe that a recommendation against slide drying plates is not warranted, since wide swings in temperature are not characteristic of all such plates. However, a statement was added to the effect that some plates have wide temperature swings. The document addresses some possible methods of drying slides, but the subcommittee does not believe it possesses the imagination necessary to exhaust the topic. The subcommittee is not in a position to provide a rigorous definition of dry.**

30. Several comments suggested that either steel or disposable microtome blades are acceptable for sectioning paraffin.

- **The subcommittee agrees and has modified this section accordingly.**

5.5.1.3 #6 and 5.5.2.2 #8 Camel Hair Brush

31. Problem: It does not matter if the debris is cleared with a camel hair brush, another type of soft bristle brush, or gauze. Suggestion: Remove "...with a camel hair brush." Have the statement read: "Rough cut the tissue sample to obtain the desired cutting plane. Clear the knife of any debris."

- **The subcommittee concurs and has modified these sections.**

Section 5.5.2.1

32. Excessive hydrochlorofluorocarbon refrigerant spray can cause some tissues to crack. A more controllable cooling process is preferred.

- **Rather than to recommend against the use of these sprays, the subcommittee has included language in this section noting that the rate of freezing associated with the use of hydrochloro-fluorocarbon refrigerant spray bottles is difficult to control.**

33. If a block is to be stored frozen it is convenient either to arrange it for freezing on a cork disc (or several layers of damp filter paper) that can be labeled on the nontissue side with ball-point pen before freezing, or to mount a prelabeled cork disc on top of the mold before freezing so that it is attached to the block by the embedding medium. The cork is an insulating layer that can be handled with forceps without danger of touching the tissue and can be frozen onto the pre-cooled cryostat object holder with a drop of embedding medium. It can be removed with a razor blade (or by heating the object holder if the cryostat has a specimen holder that is thermoelectrically controlled), and the frozen embedding medium can be scraped off to reveal the written label.

- **This is a useful suggestion that has been incorporated (with modifications) into the document.**

34. We recommend adding the following statement after the second sentence of this section: "The exposed edge of tissue should be protected with additional embedding media to avoid freezer burn during long-term storage."

- **The subcommittee agrees and has revised the text in (1).**

Section 5.5.2.2

35. "Mounted sections should be fixed in cold (-20 ^{0}C) acetone." For how long? For some antigens,

e.g., ER and PR, buffered formalin acetone pH 6.6 is recommended.

- **The subcommittee has added language addressing these issues.**

36. There are other methods for freezing tissue, such as Histobath (sold by Shandon-Lipshaw), which keeps the isopentane at –50 °C. There is also another method that used cold carbon dioxide in a canister (do not remember what company makes this). These methods could be used. Drop all mention of methods of freezing tissue.

- **The subcommittee notes that both the methods referred to by the commentor were mentioned in the proposed guideline (although without mentioning any particular vendor). We believe it is useful to alert those who have been exposed to a limited number of approaches for freezing tissue that there are a large number of useful alternatives to consider.**

37. There is usually no need to fix cryostat sections immediately. Denaturing by drying at ambient temperature for one hour or longer (overnight) before fixation is usually beneficial to the tissue structure and does not harm antigenicity.

- **The subcommittee does not have enough experience in this area. The need to fix cryostat sections immediately may be dependent upon tissue type and antigen.**

Section 5.5.3

38. Ethanol fixation can be detrimental to some antigens. Acetone could also be recommended as a robust fixative for cytologic material.

- **Subcommittee members who are practicing cytopathologists believe that, while ethanol fixation may occasionally be detrimental to antigenicity, acetone fixation is almost invariably detrimental to preservation of cellular morphology; they are unwilling to recommend this alternative.**

Section 6.1

39. In the discussion of "antigen retrieval," enzyme digestion is mentioned only in passing and on page 36 is referred to as obsolete. I have found instances where microwave digestion is inferior to protease - most recently with the keratins.

- **The subcommittee notes that the term used in the proposed document (Section 8.5.1.4) is largely obsolete and that the language of the proposed guideline states clearly that protease digestion is preferable for some antigens. There does not appear to be universal agreement on which antigens these are, however. A phrase has been added in Section 6.1 indicating that enzymatic digestion may be preferable for some antigens, to complement the statement to the same effect in Section 8.5.1.4.**

40. In practice few labs will have time or resources required to employ different antigen retrieval regimes to different antigens – a general-purpose method which works for all should be recommended.

- **The subcommittee does not believe it is in a position to recommend a single general-purpose antigen retrieval technique, because the best approach for any particular laboratory needs to be optimized to match the tissue fixation protocols in use and the immunohistochemical assays employed (both antigens and assay system).**

41. Table 2: Perhaps it would be best to recommend that the volume of fluid and number of slides remain constant, as these parameters are important for reproducibility when using microwave

oven retrieval.

- **The subcommittee members have not found that the volume of fluid matters greatly, as long as there is a great excess. We have not found the number of slides to affect reproducibility much at all.**

42. Isn't "antigen retrieval" a registered trade name? Would it also include digestion? Use a more generic term such as "antigen enhancement."

- **The subcommittee believes that the term antigen retrieval is clearly the most widely used term for this process, and hence the most easily recognized. The use of this term outside the context of any trade name has been widespread, and its use in the literature predates the attempted use as a trade name. The company that registered the term was not successful in maintaining exclusive use. As noted, the term antigen retrieval encompasses enzymatic digestion in this document.**

43. I have no experience of the steamer, but fear that retrieval is occurring in pure steam only. Hence the recommended solutions are not appropriate for this retrieval method.

- **Although it is clear that the concentration of solute in the vapor phase will be lower than that of the aqueous phase, it may or may not be zero (most of the commonly employed solutes will be absent from the vapor phase, however). In addition, these solutes may change the partial pressure of water in the vapor phase (assuming constant temperature). Although we suspect that the commentor is correct that the changes are occurring in pure steam, little study has been given to the comparison of pure water with an antigen-retrieval solution. Several subcommittee members have done such comparisons in other systems (water bath and microwave), finding that although various additives do improve antigen retrieval in these heating devices, some antigen retrieval does occur with distilled, deionized water. Unfortunately, the subcommittee has not looked at the specific case of the steamer. In the absence of additional data, we are inclined to go with the approaches published in the literature, whether or not they have been perfectly controlled or understood.**

44. One of the most popular retrieval solutions in Europe is 1 mM EDTA.

- Although it is not the subcommittee's intent to cite the extensive literature of proposed antigen-retrieval solutions, we have changed the text to include this solution.

45. "In general, usage of the term 'antigen retrieval' now describes a variety of techniques that may be applied when performing immunocytochemical staining on paraffin sections that have been less than ideally fixed." This sentence suggests that people use heat-mediated antigen retrieval to compensate for bad processing habits and that with an ideal fixation antigen retrieval will never be required! What is the ideal fixative for ER or PR 017 paraffin fixations that will enable an adequate immunostain without using heat?

- **In the opinion of the subcommittee, the ideal fixative is a fixative that preserves morphology without destroying antigenicity or recoverability of nucleic acids. In this sense, the ideal fixative has not been identified, and the statement in the guideline is not intended as a criticism of anyone s processing methods.**

46. Concerning the use of equipment intended for kitchen use that usually has a limited guarantee, e.g., microwave ovens. Are they usually guaranteed for one year? What happens after the guarantee has expired? Should you buy another one?

- **Maintenance of general laboratory equipment is beyond the scope of this guideline.**

47. More detailed recommendations, e.g., on the pH of antigen retrieval solution, could be useful. Many laboratories still use citrate pH 6 as the only solution, though Tris (-EDTA) pH 9 is more efficient with most epitopes. The problems with endogenous biotin that accompany an efficient antigen retrieval should be emphasized.

- **The subcommittee believes that it is inappropriate to provide detailed recipes in this or any other part of the guideline, because they are likely to be either outdated, disputed, or both. A comment has been added regarding potential problems with increased detection of endogenous biotin.**

48. Recommend that the laboratory follow the manufacturer's specification sheet, rather than work through the entire table of testing for antigen retrieval. There should be sufficient information on the specification sheet to have testing include the times, but not all levels of pH. Or, make this paragraph into two paragraphs, one discussing the testing required for an in-house developed antigen retrieval buffer, and one paragraph discussing commercially prepared antigen-retrieval solutions.

- **The subcommittee believes that the manufacturer s specification sheets do not always adequately account for variation in local conditions. Laboratories are, of course, free to try the manufacturer s recommendations as they are, but should not believe that they have optimized the antigen retrieval process by doing so.**

Section 6.2

49. Several commentors suggested that the commercially available charged slides should be mentioned under Slide Adhesives.

- **A reference to the availability of charged or frosted slides has been added.**

Section 6.2.2

50. Poly-L-lysine (MW 150,000 to 300,000) may also be spread in a thin layer on a slide as for a blood smear. This method is more economical, but requires experience to get a thin enough layer (interference colors seen as the drop (10 microliters or less) is pushed along the slide surface with the edge of another slide held at about 30 degrees to the first). This procedure is less time-consuming than having to rack up slides for dipping and derack them for storage. Slides are ready for use immediately and can be stored indefinitely.

- **This method of preparation has been added to the document.**

Section 7

51. Add one section before Automation that discusses the amplification technology of immunochemical staining, for example, biotinylated tyramide. This technology is available now, and is being used increasingly by clinical laboratories. A pictorial representation of this methodology could be added to Figure 1.

- **All indirect immunocytochemical assays employ some form of amplification. The biotinylated tyramide technology has been well advertised, there is no doubt that most laboratories are aware of it. It appears to be a proprietary technology, however, and the subcommittee is reluctant to give this or any other proprietary approach undue prominence within the document.**

52. Will there not be much detail on immunostaining as there is on specimen preparation? Does the titration and testing part serve to guarantee or provide guidelines for the proper performance of

the immunologic reactions?

- **The subcommittee recognizes the apparent disparity in attention given to the section on the immunological aspects of test performance in comparison with the attention given to pre-staining considerations. There are numerous published procedures for immunocytochemical testing, and manufacturers of immunocytochemical reagents will almost all gladly provide a number of different, effective procedures. In general, however, these published procedures give insufficient emphasis to the preanalytic factors affecting immunocytochemical staining, although in the experience of most subcommittee members, these preanalytic factors more frequently contribute to suboptimal immunocytochemical staining than do variations in immunocytochemical staining procedures themselves.**

53. The immunoglobulin subclass of the primary is sometimes important to note. For example the following reference indicates the need to use an appropriate bridge reagent for CD15 antibodies: LeBrun DP, Kamel OW, Dorfman RF, Warnke RA. Enhanced staining for Leu M1 (CD15) in Hodgkin's disease using a secondary antibody specific for immunoglobulin M. *Am J Clin Pathol.* 1992;97:135-138.

- **A statement on the importance of careful selection of the secondary antibody has been added to this section.**

Section 7.1

54. I have no experience with avidin-labeled secondary antibodies in routine use; there is no mention of biotinylated secondaries - i.e., the ABC method as I understand it (Fig. 18 on pg. 17 does show the ABC method correctly).

- **This oversight has been corrected in the text.**

55. Figure 1: This diagram can be somewhat misleading, since it appears to be intended as a general schema of methodologies. The misleading portion is the term "enzyme" or "E" in the illustrations. This should be "Label" or "L," since the general mechanisms illustrated can use either enzyme labels or fluorescent labels. The title of this figure is also somewhat misleading, since the figure is an incomplete representation of the "methods" of immunostaining. The problem is that each diagram is incomplete. There is no mention anywhere of the visualization of the label, i.e., enzyme substrate chromogen.

- **The subcommittee agrees that, while the illustrations could be used to help describe immunofluorescent assays, we consider these to be outside the scope of this document. However, we added a note to the figure to indicate that visualization of antigen occurs when enzyme reacts with its substrate, enabling deposition of chromogen.**

56. More detailed recommendations concerning the methods of choice for high sensitivity may be useful. The new dextran polymer techniques should be included. (See Vyberg M, Nielsen S. A dextran polymer conjugate two-step visualization system for immunohistochemistry: a comparison of EnVision+ with two three-step avidin-biotin techniques. *Appl Immunohistochem.* 1998 6:3-10).

- **The subcommittee is unwilling to make specific recommendations of this sort, both because they may become quickly outdated, and because improvements in the sensitivity for detection of a specific analyte are not necessarily reflected in improved diagnostic utility.**

57. Diagram–Outline the alkaline phosphatase-antialkaline phosphatase method and possibly EnVision.

- **As given, the diagram is quite general and does not specify any particular enzyme. Hence, the alkaline phosphatase methods are included. The subcommittee does not believe it s appropriate to outline the proprietary technology, which has been well illustrated in company advertisements.**

Section 7.1.1

58. There are too many variables in the staining procedures used. Direct vs. indirect. Using endogenous blocking or not, depending upon chromogen used. Use of antigen enhancement or not. Counterstain time, which depends upon which counterstain was used, the concentration, and pathologist's choice. Drop this example. There is no need for an example.

- **The subcommittee agrees that there is much room for variation. Of the many comments received, this was the only one that suggested dropping this section, suggesting that most people found it to be useful. We believe that it may assist the novice to realize that there is some room to modify immunocytochemical procedures to improve results, and that it should be retained if only for this reason.**

59. The use of a humid chamber for slide incubation is not mentioned, but should be because of the danger of drops of reagent drying on the slide.

- **This recommendation has been added to Section 7.1.1.**

60. Many people in this country incubate overnight at 4 °C with the primary antibody, particularly if it is a polyclonal which would benefit from increased dilution to avoid background staining.

- **The proposed guideline mentions the use of longer incubation times at 4 °C in Section 7.2.3.**

Section 7.1.2

61. End of last sentence of 2nd paragraph: what is a "stat" staining procedure?

- **The term stat is widely used in North America to refer to a laboratory procedure which returns a clinical result very quickly - usually within an hour or less. The sentence was modified to make this clear.**

Section 7.1.3

62. End of last sentence of 1st paragraph: One could have the impression that, in order to compare a manual technique and an automated technique, a pathologist has to conduct a parallel testing of both methods and judge the impact of staining on clinical parameters!

- **This is exactly the intent of this recommendation. If the two methods give identical results, then no issue of clinical effect of the change is raised. If, however, the introduction of the automated technique seems likely to give rise to changes in diagnosis in substantial numbers of patients, the clinical impact of the change must be carefully considered during the implementation phase.**

63. Manufacturers are obviously the best advisors for machine operation and maintenance, but some offer inappropriate guidance on immunocytochemical techniques. For example, prediluted primary antibodies are often set at dilutions that are not appropriate for local conditions. More often than not the primary reagents are too dilute.

- **The subcommittee does not have the data to justify such a statement in this document. If an individual user is experiencing this problem, it is appropriate to take it up with the instrument manufacturer and, if appropriate, regulatory agencies.**

Section 7.1.4

64. "A positive and two negative controls should be used for tissue controls." In France, many people think that this requirement can be fulfilled in many cases by using internal controls within patient specimens. Only if this requirement cannot be fulfilled by using internal controls will external positive and negative control be included in the technique. Is this way of proceeding acceptable according to the proposed guidelines?

- **The use of internal controls is generally acceptable, and may be preferable as a result of cost-containment considerations.**

Section 7.2.3.1

65. Use of secondary antibodies at different dilutions for each primary would be practically impossible and of little benefit; a single dilution needs to be established by the user.

- **Use of a single dilution is convenient and is usually acceptable (as with commercial preparations of prediluted secondary antibodies). Nevertheless, somewhat improved results may often be obtained by simultaneous optimization of the concentration of both primary and secondary antibodies. As suggested by the commentor, this is a lot of work; it is up to the individual laboratory to determine whether the benefits are worth the efforts.**

Section 7.2.5

66. Surely the sprayed buffer must contact the section? Do they mean (rather obviously) that the plastic tip of the wash bottle must not touch the section, or do they mean that the buffer should be sprayed onto the slide and more gently tilted towards the section? This should be clarified.

- **This statement has been clarified in the text to suggest that the buffer should be sprayed onto the slide and allowed to wash gently over the tissue section itself.**

67. Some workers and all instruments employ surfactants in the wash buffers, e.g., Tween 20.

- **The text has been modified to note this fact.**

Section 7.2.6

68. The unlabeled antibody method is mentioned twice. Does the second mention need to be "the enzyme-antienzyme complex method (e.g., peroxidase-antiperoxidase)"?

- **Yes. The text has been so modified.**

69. Paragraph 4 is not correct in my understanding of the enzyme-antienzyme method. I think the enzyme-bridge technique has been combined with the enzyme-antienzyme technique, which has confused the description. It should read something like: In the enzyme-antienzyme technique the primary antibody binds to the antigen in the specimen. The secondary antibody binds to the primary antibody and forms a bridge between the primary antibody and the third reagent which is a preformed complex of an enzyme with its antibody. The antienzyme should be from the same species as the primary antibody (Fig. 1.4). In an alternative method, the enzyme bridge technique, (Fig. 1.3) the antienzyme (same species as primary antibody) is applied as a third layer followed by the enzyme.

- **Paragraph 4 has been rewritten to be clearer.**

70. The title of this section appears to be inconsistent with the subject matter.

- **We have retitled the section Visualization Methods.**

71. We recommend including the use of streptavidin, since it is quite popular and more sensitive than avidin.

- **We have included text that mentions streptavidin as an alternative to avidin, but do not believe it is necessary or appropriate to recommend the use of one over the other.**

72. Paragraph 3 needs to be rewritten. First, it is questionable if peroxidase is the most commonly used enzyme label in Europe. The other problem with this paragraph is that it confuses the use of peroxidase enzyme label with peroxidase-antiperoxidase technique, and alkaline phosphatase enzyme label with alkaline phosphatase-antialkaline phosphatase technique.

- **This paragraph was extensively rephrased.**

73. Paragraph 4 should start off with "Enzyme labeling techniques employ a 1° antibody, linking or 2° antibody, and a mechanism for attaching the enzyme label to the secondary antibody. The 1° antibody binds to the antigen in the specimen, and the secondary antibody (which reacts with the species of the 1° antibody) forms a link or "bridge" between the primary antibody and the enzyme labeling system (see Figure 1). The most commonly used enzyme labels for paraffin section immunostaining are peroxidase or alkaline phosphatase."

- **Paragraph 4 has also been rewritten to make it clearer.**

74. This section does not define the most popular methodologies currently in use, which is simply the avidin biotin (or strepavidin) linking system. This is not the same as the ABC method, which is based on a preformed complex of avidin and biotin molecules. An additional paragraph needs to be added to address this omission.

- **The subcommittee does not know for certain what is the most popular method in current use, but believes that the chemical differences between preformed complex and nonpreformed complex ABC methods are sufficiently inconsequential as to not warrant distinction in this document.**

Section 7.2.7

75. We recommend a warning statement related to the reported carcinogenicity of DAB and AEC.

- **The subcommittee has included language to this effect in Section 4 (Safety)**.

76. The substrate for peroxidase is hydrogen peroxide and not DAB or AEC.

- **The word substrate has been changed to chromogen in this sentence.**

77. This is far too simplistic an approach to multiple labeling which is fraught with problems of cross-reactivity. It would be enough to say that different chromogens can be used in multiple labeling techniques to give a differently colored end product for localization of different antigens. Maybe provide a reference for multiple-labeling techniques and say it is complicated.

- **We have added a statement noting the complexity of multiple-label techniques, and commenting that they are seldom employed in routine clinical practice.**

Section 7.2.8

78. Use of the word "halftone" is incorrect. The correct term should be "monochrome."

- **The word halftone has been changed to black and white for clarity.**

79. Several observers suggested that hematoxylin should be recommended more highly as a counterstain (provided the staining is light), suggesting that the methylene blue counterstain is not widely used.

- **The subcommittee agrees that a light hematoxylin counterstain is adequate for most immunocytochemical assays, but believes that the description of the positive and negative aspects of each of the counterstains discussed in this section is correct.**

80. It should be mentioned that methyl green is water-soluble and of no use with aqueous mountants.

- **A note to this effect has been added.**

Section 7.2.9

81. Table 4: Several commentors noted that CD3O does not stain plasma cells.

- **The table has been modified accordingly.**

Section 7.2.9.1

82. The mention of vimentin as a fixation control is interesting, since there are people who would rather test for vimentin itself using antigen retrieval (which increases reactivity tremendously), which would counteract a control purpose. This debate is ongoing.

- **The subcommittee acknowledges this comment.**

83. If all antibodies are appropriately stained throughout the tissue section, there is no need for the use of a vimentin control. The patient's tissue IS a good indicator of processing quality. If the staining is uneven, or if all antibodies are not staining, this might indicate a problem with staining, or possibly with the processing/fixation. In either case, if the antibody staining were uneven or inadequate, the case would be restained. At that time, it would be appropriate to run a vimentin control, to see if the problem of uneven staining or lack of staining is due to a fixation/processing problem. To run a vimentin control on every case is a waste of reagents and of patient's tissue, and adds to the cost both in reagents and time. Rewrite this area to reflect the above, i.e., that vimentin should be used as a control when the staining of antibodies is inappropriate, uneven, or inadequate.

- **The subcommittee s intent is not to suggest that vimentin needs to be used routinely as a tissue processing control, but rather to point out some of the strengths and deficiencies of this popular approach. The first sentence of Section 7.2.9.1 has been reworded to eliminate this confusion.**

84. Vimentin is one of the easiest antigens to stain after heat-mediated antigen retrieval. Hence not much information will be deduced in that situation.

- **The subcommittee agrees that the presence of staining is not terribly informative in this case, but the lack of staining carries substantial information. For this reason, the subcommittee believes the vimentin control is often worthwhile.**

Sections 7.2.9.1 and 7.2.9.1.1

85. I believe these sections are out of date, now that heat-induced antigen retrieval is well established. Haematopathology cases usually require some T- and B-cell markers as a matter of routine. The quality of staining of these antigens will usually act as a guide to the quality of fixation and processing. If, however, heavy and light chain immunoglobulin staining is required, then the quality of fixation is very readily seen. Poor fixation will show very diffuse staining with poor localization, whilst the staining in optimally fixed samples will be much crisper and better localized.

    In most other diagnostic areas too the antibody panels relevant to the diagnosis will give some indication of the viability of the tissue. For instance, ER staining is best judged by the staining of the internal normal glands. Sometimes ER protein is lost because of delays in fixation, but the vimentin epitopes are often still retained.

- **The subcommittee does not mean to suggest that vimentin is the only alternative for processing QC, and has reworded these sections to make this more clear.**

Section 7.2.9.1.1

86. Immunoreactivity for vimentin is not parallel with that of estrogen and progesterone receptor; duration of heating needs to be longer for ER or PR than for vimentin.

- **The subcommittee recognizes that not all laboratories will obtain identical results, but believes that the statement appearing in this section reflects the experience of most laboratories performing immunocytochemical assays for ER and PR.**

Section 7.2.9.2

87. In most institutions, it is not feasible to obtain tissues with a range of reactivity for many of the antibodies. Also, to obtain these tissues often requires destroying the patient's blocks. For most institutions, it is not cost-effective to purchase controls with a range of reactivity. Plus, purchased controls have NOT been fixed and processed the same as the institution's tissues. This again could lead to many problems. The main problem is the use of extremely positive controls, especially the use of normal tissue. This can lead to a false-negative reaction of a patient's tissue that is displaying weak-to-moderate reactivity. Rephrase the statement to reflect that an ideal control would NOT be extremely positive, would be similar in reactivity to the patient's tissue, and, if at all possible, should contain tumor tissue, not normal tissue.

- **The subcommittee concurs with the commentor s belief that if control tissue with a range of reactivity cannot be obtained, the control tissue that is used should be one that is only weakly reactive for the antigen under consideration. Nevertheless, the subcommittee also believes that it is generally straightforward to construct multitissue blocks having a range of reactivity, with little damage to patient care material. Finally, we believe that, while use of purchased control materials may sometimes be necessary, those prepared in the home laboratory more accurately depict the conditions to which any new tissue is likely to have been exposed.**

88. I have not come across this problem of deteriorating antigenicity in practice. Can the authors give any examples of antigens that might be affected?

- **Subcommittee members have seen this with PCNA, B72.3, EMA, ER, PR, and others.**

Section 7.2.9.3

89. The "negative tissue control" seems to me to be uninformative. The primary antibody should already have been established as giving minimal spurious background staining, and the level of background staining on the test specimen due to tissue factors should be obvious from the nonspecific staining control (Section 7.2.9.4). A known positive control will ensure that the antibody is staining correctly.

- **The subcommittee believes this control is almost always present within the slide that is being tested, and so indicated in the proposed guideline with the sentence, The variety of different cell types present in most tissue sections offers internal negative control sites. The subcommittee members do not believe that this interpretation differs from the intent of the commentor s remark. However, to make the point we used a bold typeface for this sentence.**

90. I am not certain that a negative tissue control is absolutely necessary in all cases. The haematopathologists will usually look at a panel of antibodies. Each antibody in the panel will have an appropriate positive control such as a tonsil or Hodgkin's disease section. The T- and B-markers will be almost a mirror image of each other (in tonsil), whilst the Hodgkin's markers such as CD15 & CD30 recognize cells with very distinct morphology. In all control slides there should be a series of negative cells which would act as a negative control. A negative tissue control would not add any further information and hence there is an argument that it should possibly be introduced only when there is some doubt.

- **The subcommittee agrees. Please see the response to the previous comment.**

91. Why are two negative controls required? No explanation is given.

- **Only one of these controls may sometimes require a separate slide; the other can almost always be performed by examination of cellular elements found near the tissue of interest, within the same tissue section. Please see the response to comment 89.**

Section 7.2.9.4

92. It is not feasible to use a negative reagent control that "contains the same isotype as the primary control, produced from tissue culture supernatant in the same way as the primary antibody... (and) is diluted to the same immunoglobulin or protein concentration as the diluted primary antibody using the identical diluent." The time it would take to prepare all the different diluents, the cost of the solutions, and the number of patient slides needed so that each antibody diluent is represented, make this unfeasible. Use of FA buffer or PBS should also be acceptable as the negative reagent control.

- **While the subcommittee realizes that this suggestion is expedient, we do not believe that it constitutes best practice. We believe that it is prudent to recommend this type of negative reagent control, and note that most of the subcommittee s members use this type of reagent control in our own laboratories.**

Section 7.2.9.5

93. Case tissue and positive control can be placed on the same slide.

- **The subcommittee recognizes that some laboratories adhere to this practice. Although this assures that the control tissue is treated exactly the same way as the tissue of interest, the subcommittee does not believe this practice is advisable, since a pathologist examining the same**

material in the future may not necessarily be able to determine which tissue is the control, and which is the tissue of interest. If indelible markings etched on the glass slide make this obvious, then the subcommittee s concerns are likewise obviated. Nevertheless, we do not believe it is prudent to recommend the practice.

94. Table 5 — Positive control/Reaction tested: It is not the specificity but the reactivity of the antibody that is tested here. The specificity must have been established previously.

- **The subcommittee agrees, and the table has been modified.**

95. Negative tissue control/Reaction tested: This will only test for cross-reactivity if a cross-reacting antigen is present. Even if it is present, it may not be present in the test tissue.

- **The subcommittee agrees with these observations, considering them obvious, but does not believe that they call for a modification in text or table. The subcommittee believes that any such modification would either be extraordinarily long, confusing, or both.**

96. "To differentiate endogenous enzyme activity or nonspecific binding of enzymes from specific immunoreactivity, additional patient tissues may be stained exclusively with substrate-chromogen, or enzyme complexes... and substrate-chromogen, respectively." The problem with this would be the "may be stained" portion of the statement. Some techs and inspectors may think that they "must" do this procedure. Endogenous peroxidase and/or nonspecific staining controls are only needed when there is a problem. Most institutions use a protein blocking, biotin blocking, or hydrogen peroxide to remove the potential for background staining. Place more emphasis that using these controls is only for cases where there is a problem. State that with appropriate blocking of the patient's tissue for endogenous peroxidase or nonspecific staining, in most cases, separate endogenous enzymes and/or nonspecific background controls would not be needed.

- **Subcommittee members have observed many cases in which the various blocking approaches mentioned by the commentor have failed to eliminate background staining, which would have been difficult to differentiate from specific staining without the use of a control slide.**

97. It could be clearly stated that in routine diagnostic practice, it is rarely needed to run three or more extra slides for controls. "When panels of antibodies are used on serial sections, the negatively stained areas of one slide may serve as a negative control/nonspecific binding background for the other antibodies." So you do not need to run a nonspecific reagent control. Also, as stated (page 25, Section 7.2.9.3, “Negative Tissue Control”): "The variety of different cell types present in most tissue sections offers internal control sites..." In other words, most of the time, if the section to be tested is carefully chosen, it can include normal tissues with a variety of cells. Some of these cells will serve as a negative tissue control. Hence, no extra negative tissue control is needed. But also, some of these cells will serve as a positive tissue control. Therefore, no extra positive tissue control is needed. In routine diagnostic practice, you will have to run extra slides if an internal positive tissue control is not available: because it never exists in normal tissues (e.g., CMV, Herpes, Helicobacter pylori...) or because it is not present in the test tissue (e.g., thyroglobulin, prostatic markers, epithelial markers, melanoma markers in a lymph node metastasis), or if an internal positive tissue control is not suitable.

- **Subcommittee members have noted that there are two conflicting sources of pressure facing the laboratory testing community regarding the use of controls. One source of pressure is from budget and financial officers, who wish to reduce the use of controls to save money. The second is from quality assurance coordinators and laboratory inspectors, who wish to clearly specify the types of controls which must be used. We do not wish to weigh in on either side of this argument. The subcommittee believes that a carefully trained immunocytochemist realizes that controls must be used, and that sometimes these controls can be internal to the patient**

specimen, and other times they are not. He/she also understands well the regulatory and financial constraints operating locally, as well as the local patient care requirements. The subcommittee does not possess the collective imagination necessary to exhaustively enumerate the various possibilities, but strongly believes that the well-trained immunocytochemist/pathologist will be able to ascertain on a case-by-case basis what is required. We further believe that the immunocytochemist/pathologist must be able to explain to the reasonably intelligent layperson or clinician the controls that were employed in an individual case.

The nonspecific reagent control that is defined in the guideline is said to be ideal. The subcommittee strongly believes it to be superior to the use of other forms of nonspecific reagent control and recommends its use if local conditions permit. The subcommittee further notes that this type of control may sometimes be difficult to obtain.

Section 7.3

98. 3% hydrogen peroxide seems quite strong. We normally use 0.3% to 0.6% hydrogen peroxide.

- **This is a typographical error which has been corrected.**

99. Should guidance about I endogenous enzyme blocking methods be given?

- **Some additional comments about blocking have been added in Section 7.1.1, with references to Section 7.4.**

100. More details of microwave output and volumes of fluids are required.

- **The subcommittee members have found sufficiently wide variations in laboratory practice, all of which seem to give good results; the subcommittee does not believe it can reasonably provide more detail. Reference to the published literature, together with experimentation in the laboratory, can generally provide a solution to this problem within a day, once a heating method has been selected. The choice of heating method is based largely on convenience.**

Section 7.4

101. Several commentors offered useful suggestions for improving the troubleshooting suggestions, including:

Weak or no staining of all slides:

- Incorrect dilution of reagents
- Buffer pH incorrect
- Degraded hydrogen peroxide.

Control slides stain, but there is no staining of specimens:

- Antigen may not be masked but could be absent.

Excessive background on all slides:

- Chromogen deposits due to high titer of primary or secondary antibodies.

- **The subcommittee agrees with these suggestions and has incorporated them.**

Section 8

102. As a surgical pathologist working in a routine clinical lab, I find it difficult to find what is applicable to my practice in this chapter. Some examples:

- I purchase for the first time the antibody (cocktail) to LCA. This antibody has been used worldwide as a general marker for leukocytes and lymphomas. Do I need to investigate hundreds of sections before I can use it routinely? Or do I just need to check whether I can make it work satisfactorily, after testing three normal tissues and three lymphomas?

- I have been using a monoclonal to PSA for two years and buy a new lot from the same manufacturer. The technical sheet indicates that the concentration has been slightly changed, but the range of suggested dilutions for immunohistochemistry remains the same as previously. How many slides to I need to validate this new lot? Can I just cut sections from three prostate cancers with three dilutions?

- I recently bought a new vial of a monoclonal antibody to cytokeratins (same clone, same 104, same manufacturer). Do I have to validate it?

- I think that this chapter should clearly make a distinction between different situations:

  — Situation 1 Validation of an antibody freshly produced and not previously tested: this area concerns more manufacturers and researchers than practitioners; validation needs to be comprehensive, because the purpose is to obtain the validation of the diagnostic specificity or prognostic value of the antibody.

  — Situation 2 Validation of a monoclonal antibody available through a commercial firm operating with a stringent quality system (ISO 9000 or equivalent), which has been thoroughly tested and published by expert teams: validation here concerns the ability of the lab to make the antibody work effectively; usually a limited range of controls will be tested, mostly positive tissue controls.

  — Situation 3 Validation of a new clone against a well-known antigen: if this antibody has not been thoroughly tested/published by experts, validation should be more comprehensive than in Situation 2 and should include tissues/tumors expected to be negative.

  — Situation 4 Validation of a new sample or a new lot of a primary antibody that has been used for several years in the lab: an easy thing to do is to immediately test the new vial of antibody in parallel with the old vial, before the latter is empty, just to check on a control (!)positive and negative tissue control) whether the level of staining remains the same or not. For example, a section of appendix can be used to test for LCA, cytokeratins, desmin, muscle actin, 51 00, chromogranin, synaptophysin...

- **The subcommittee does not believe it is either prudent or possible to attempt to exhaustively discuss various individual circumstances. Most subcommittee members are not inclined to attempt to provide different sets of guidance for slightly different situations, each of which still amounts to implementing a previously unused reagent into the laboratory. We do not believe that the suggestion that new reagents be validated on large tissue panels has been unreasonable since the advent of multitissue blocks. Multitissue blocks are easily prepared; a preparation of three or four such blocks is likely to give sufficient tissue for assay validation within the laboratory for several years. Manufacturer testing does an excellent job of determining how an immunohistochemical assay will perform on tissue from somebody else s institution. With the exception of the few individuals involved in validating these assays in cooperation with the manufacturer, more extensive testing is required within the individual laboratory for whom**

fixatives and fixation conditions may vary tremendously from those that were employed by the manufacturer. With multitissue blocks, it is not extraordinarily time-consuming to perform this level of validation on tissue representative of that with which the laboratory will be dealing daily.

We do not consider a new lot to require complete revalidation. Appropriate checkerboard titration is dealt with in Section 7.2.3. The subcommittee considers this level of titration to be optimal for checking the performance of new lots of previously characterized antibodies.

103. Add a section that contains guidelines for ready-to-use antibodies.

- **The subcommittee agrees that this is worthy of comment, and has added a short section (Section 8.7) to deal with test kits.**

Section 8.2.1.2

104. Strictly speaking 'avidity' refers to the force of binding associated with multivalent binding with a single reagent, e.g., a bivalent antibody binding with both its site and its target antigen (cf 'affinity' which relates to the force of binding associated with a single site or univalent binding reaction). The functional affinity resulting from a polyclonal antibody reflects the average affinity/avidity of all the individual variety of specific antibody molecules interacting with the same target antigen either univalently or multivalently. The interaction of a polyclonal antibody with its target antigen may therefore be defined to be 'polyvalent' rather than 'multivalent,' as a way of distinguishing the two types of interactions.

- **The text has been modified.**

Section 8.3

105. There is a recommendation to use the Biological Stain Commission guideline for the development of a manufacturer's specification sheet. The manufacturer is now also constrained by the Food and Drug Administration, Center for Devices and Radiological Health to use the draft guidance package insert dated 3/27/95. It may be best to recommend that either of the guidelines be followed.

- **The subcommittee has modified this section to take into account documents that may be promulgated by the U.S. FDA or other government agencies.**

Section 8.5.2.8

106. I disagree with Section 8.5.2.8 in that some markers may be tested on tumors arising in some of these tissues where you wouldn't expect to find the marker, and a history of reactivity is not necessarily irrelevant.

- **The subcommittee is uncertain of the intent of the comment, but notes that the intent of the panels is to suggest reasonable tissues to consider in the process of assay validation. Depending on individual circumstances, it may be appropriate to either broaden or narrow the range of tissues included.**

Sections 8.5.2.4 through 8.5.2.9.4

107. What exactly is meant by "panel" in these examples? Does it mean that one control MUST have all the various tissues listed, whenever doing a stain for those tissues, i.e., if doing any neuroendocrine stains, the control must have the ten tissues listed? Does it mean that if running a stain for any one antigen (e.g., insulin), that ALL the tissues listed under neuroendocrine must be also run? If either of these are the case, then for most institutions,

this is not feasible, practical, or economical. Better define what is meant by a "panel." Then instruct the technologists to chose the appropriate control for the antigen being run.

- **Section 8 is devoted entirely to validation of new or modified immunocytochemical assays. The sections referred to by the commentor are not intended to pertain to the routine performance of immunocytochemical testing, which is dealt with in Section 7. The subcommittee believes that more than a single control is required in this initial validation phase.**

Section 8.6

108. Most institutions do not wait three to six months after introducing a new antibody, to assess how it is performing. Most problems are seen and dealt with during the assessment stage. The antigen is not placed in the repertoire of the institution until after all the problems have been dealt with. Once in place, IF any problems arise, they again are handled as soon as they arise. Institutions should constantly be assessing ALL antigens, new and old, as to methods to increase their effectiveness. This is part of the quality assessment program of any immunohistochemistry laboratory.

- **The subcommittee believes that most laboratories continue to discover new aspects of reagent performance long after a new assay is introduced, and that this discovery process is facilitated by a formal postintroduction surveillance period. Although we agree with the statement that institutions should constantly assess all antigens, we believe that this assessment is often quite haphazard and ineffective in many laboratories.**

109. In most institutions, this number is not feasible. Many institutions may not have 15 to 20 cases of the particular tumor, or the same number of similar tumors that may be confused with it.

Suggestion: Drop the numbers 15 to 20, and leave it more generic, such as testing "numerous" cases in both categories. Let the institution determine the appropriate number for their laboratory, the cases available, and the type of antigen. The institution would have to prove that the number of cases they chose to use, both with the antibody and those that could be confused, was adequate to assess the new assay.

- **The subcommittee understands the commentor s concern, and notes that this was a matter of substantial discussion within the subcommittee. Nevertheless, the subcommittee believes it is more useful to the laboratorian to provide specific guidance, based on statistical reasoning, in this case, rather than leave the numbers totally up in the air. The subcommittee further realizes that individual laboratories will have to adapt all of these guidelines to fulfill their specific clinical and regulatory requirements.**

Section 9.3

110. Several individuals commented that it was laborious and unnecessary to place information on lot numbers and source of the antibody in report.

- **The subcommittee agrees, and has modified the text to show that this documentation should be maintained by the laboratory.**

111. Basic information on the immunostaining protocol could be briefly reported, e.g., ABC peroxidase on paraffin sections after heat-mediated antigen retrieval in the report.

- **The subcommittee believes that this is an acceptable process, but should not be specifically recommended.**

Section 9.4

112. Is it possible to discuss the expiration dating of antibodies? Consider freezing the antibody in –70 °C as an aliquot for a prolonged period. Extend the expiration date, since tissue control provides evidence that the antibody still works.

- **The subcommittee recognizes that, as with other analyte-specific reagents, it may be possible to use antibodies after their initial expiration date. The prime method for determining suitability is, as suggested by the commentor, appropriate reaction with control tissue and appropriate behavior in everyday use. In the opinion of many subcommittee members, the use of appropriate controls makes the expiration dating of antibodies a practice to which we conform for regulatory reasons, rather than because it assures better patient care. The regulatory environments vary greatly within the intended audience of this document, and the subcommittee does not believe it can adequately address this variation within the body of the document. Hence, we feel compelled to restrict our input on this vexing matter to this comment.**

Section 9.4.1

113. There is little significance in reporting intensity of staining for the immunoperoxidase procedures. Remove this requirement of stating in the patient's report the intensity of staining and the controls used to assess the intensity.

- **The subcommittee agrees that in some cases the intensity of staining need not be reported, but that in other cases this is very important information. In the subcommittee s opinion, the wording of this section makes it clear that the report may include information on intensity of staining.**

Related NCCLS Publications*

GP2-A3 **Clinical Laboratory Technical Procedure Manuals—Third Edition; Approved Guideline (1996).** This guideline addresses design, preparation, maintenance, and the use of technical procedure manuals in the clinical laboratory.

GP5-A **Clinical Laboratory Waste Management; Approved Guideline (1993).** GP5-A provides guidance on safe handling and disposal of chemical, infectious, radioactive, and physical waste generated in the clinical laboratory.

GP15-A **Papanicolaou Technique; Approved Guideline (1994).** GP15-A discusses procedures for cervical specimen collection, as well as the preparation, fixation, staining, and storage of Papanicolaou slides.

M29-A **Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue–Approved Guideline (1997).** A consolidation of M29-T2 and I17-P, this document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

MM1-P **Molecular Diagnostic Methods for Genetic Diseases; Proposed Guideline (1997)**. This document provides guidance for the use of molecular biological techniques for clinical detection of heritable mutations associated with genetic disease.

MM2-A **Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline (1995).** MM2-A provides guidance for conducting molecular tests of immunoglobulin and T-cell receptor gene arrangements.

MM3-A **Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline (1995)**. MM3-A is a guideline for the use of nucleic acid probes and nucleic acid amplification techniques for the detection of target sequences specific to particular microorganisms. It also addresses quality assurance, limitations, proficiency testing, and interpretation of results.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

Related NCCLS Projects in Development

MM5 **PCR-Based Assays for Molecular Hematology.** This guideline will address the performance and application of assays for gene translocations by both PCR and RT-PCR techniques and include information on specimen collection, sample preparation, test reporting, test validation, and quality assurance.

MM6 **Quantitative Molecular Diagnostics for Infectious Diseases.** Recognizing the increased use of quantitative molecular methods for determining the infectious load of organisms in patients, this project will lead to a guideline on development, validation, assessment, and implementation of nucleic acid quantification assays.

MM7 **Fluorescence *In Situ* Hybridization (FISH) Methods for Medical Genetics.** This project will address FISH methods for medical genetic determinations, identification of chromosomal abnormalities, and gene amplification. Topics to be addressed include probe and assay development, qualification, and validation; instrument requirements; quality assurance; and recommendations for evaluation of results. Guidance for assay development will include detailed recommendations on specimen selection; handling and treatment; hybridization conditions/efficiency; and test limitations, precautions, and warnings.

MM8 **Measurement and Interpretation of Trinucleotide Repeats.** Several human diseases are known to be related to the expansion of trinucleotide repeats in germ-line DNA. Diagnosis of these diseases and identification of presymptomatic/unaffected carriers of unstable trinucleotide expansions require meticulous attention to accurate measurement of molecular size of the repeated segment of the gene or chromosome. A standard for the laboratory evaluation of these conditions and their carrier states would include specimen collection and handling, accuracy of molecular sizing methods, and interpretation of the sizing data. Since many applications of molecular methods in forensic and parentage analysis are based on quantitative blotting of repeat sequences showing RFLP variability, the groundwork for much of the standardization of quantitation of repeats in DNA has been well laid out.

NOTES

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