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Advances in standardization of immunohistochemistry

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Tissue fixation and antigen retrieval

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Introduction

During the last two decades, immunohistochemistry has become the most useful adjunctive method in diagnostic histopathology. The standardization of immunohistochemistry, however, has lagged behind. The quality of immunostaining depends on three major factors in addition to antibody quality: i) tissue fixation and processing; ii) unmasking of epitopes; and iii) sensitivity of the detection system (1). The fixative most often used is formaldehyde, due to its low cost, simple preparation, and because it preserves morphological detail with minimal shrinkage artifacts. However, formaldehyde fixation may result in a variable reversible loss of epitopes by masking some antibody binding sites. Although epitopes concealed by formaldehyde can be recovered using several epitope retrieval methods, the immunohistochemical detection system must be sensitive enough to give a strong signal.

Principles of formaldehyde fixation

Formaldehyde induces the formation of cross-links between proteins, or between proteins and nucleic acids, involving hydroxymethylene bridges (2, 3). Additionally, coordinate bonds may be formed for calcium ions which are the most abundant ions in tissues (4). The newly introduced cross-links may be responsible for the masking of epitopes by altering the three-dimensional structure of proteins. Cross-linking by formaldehyde needs at least 24 h to be completed (5). Shorter fixation times interrupt the formalin fixation process, which may be completed by coagulation fixation, during tissue dehydration by alcohol. Unfortunately, this results in a mixture of cross-linking and coagulation fixation.

Principles of tissue processing

After formaldehyde fixation, the tissue has to be further processed tor paraffin embedding. This includes dehydration by graded ethanols, washing out the ethanol, and incubating in warm and fluid paraffin. Tissues are then blocked in the cooling paraffin.

Principles of epitope retrieval

Epitopes that have been concealed by formaldehyde fixation can be demasked resulting in a reconstruction of their three dimensional structure. The most popular methods for antigen retrieval are proteolytic pretreatment or heat-induced epitope retrieval. Since the cleavage sites of the proteases are nonspecific, the epitopes themselves may be affected. The major disadvantage of this technique, however, is that titration of the incubation time and/or enzyme concentration is necessary (6). The modern concepts of epitope retrieval combine heat and a buffer (7), however the principles of this method are not fully understood. The heat applied to the tissue sections may provide the energy necessary to break cross-links that have been formed during the formaldehyde fixation. The buffer in which the sections are incubated during the heating process either precipitates or chelates the released calcium ions (4).

Recommendations for standardization

Delay of fixation

Delayed fixation results in an increasing proteolytic degradation which may cause irreversible weak or absent staining, or even nonspecific binding to unrelated molecules. To prevent autolysis, fixation has to start soon after surgical removal of the tissue (<30 mm). Organs and solid tumors should be incised to ensure rapid onset of fixation. A representative portion of tissue can be fixed immediately upon receiving the specimen.

Short formaldehyde fixation

As mentioned above, formaldehyde fixation of less than 24 h results in a mixture of formaldehyde and ethanol fixation with cross-linking at the periphery of the tissue block, and predominantly coagulation towards the center. Paraffin sections from such tissues may show more intense staining of the center or of the periphery, depending on the antibody applied and whether epitope retrieval was used or not. Therefore, specimens destined for immunohistochemistry should be trimmed thinly (about 3 mm) and fixed at least overnight, If paraffin-embedded tissue blocks are underfixed, the tissue can be deparaffinized and subjected to additional formaldehyde fixation to complete cross-linking and be reembedded.

Prolonoed formaldehyde fixation

Prolonged formalin fixation can lead to excessive cross-linkage which results in weak or absent immunostaining. This effect depends largely on the susceptibility of individual epitopes. Moreover, contaminating substances in the fixative probably may lead to irreversible damage to some epitopes. For these reasons, overfixation should be avoided altogether. Increasing the intensity ot the epitope retrieval step may unmask some antigens affected by prolonged formaldehyde fixation.

Tissue processing

Tissue dehydration may be insufficient if the graded alcohol solutions have been used for too long, resulting in weak or absent staining of the target cells. At the same time nonspecific reactivity may develop, especially at the edge of the sections. Contaminants in the xylenes used for defatting of tissues may also contribute to variations in the immunostaining procedure. Depending on the antigen, variation in the temperature of the wax may be deleterious.

Artifacts due to tissue processing can be avoided by preparing the soiutions used for these procedures fresh every week (or more often, depending upon the volume of tissue processed). It is very important that the infiltration processor be maintained regularly. Use of high quality xylenes or redistilling of solvents often improves their quality. The heat of the paraffin during embedding should not be overly high (<56 oo) to avoid loss of sensitive antigens.

Ealtove retrieval

If the tissue has been formaldehyde-fixed and processed appropriately, the quality of immunostaining depends on epitope retrieval and the sensitivity of the defection system applied. Since the laboratories use variably sensitive detection systems (e.g. ABC, APAAP, different dilution and chromogens), standardization of epitope retrieval on an interlaboratory basis may be difficult. For heat-induced epitope retrieval, the situation is very complex, since different buffers and pH, and to a lesser degree, different heating sources, may give variable results in individual antigens (1, 8). As a starting point, lists of which antibodies are helped by heat-induced epitope retrieval can be obtained from the literature (1). To determine the optimal conditions for different antibodies, the "test baffery' screening proposed by Shi *et al.* (9) is recommended.

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Comparative immunostaining among 15 different detection systems

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Objectives

Our objectives were to test different commercially available immunostaining kits to evaluate the results with different antibodies at different dilutions.

Methods

Consecutive sections of different tissues, formalin fixed for 6-10 h, paraffin routinely processed, were immunostained for eight widely used antibodies (Table 1); antigen retrieval was performed using a pressure cooker or proteolytic enzyme according to the experience in our laboratory.

The staining was performed using the following 15 commercially available kits: Biogenex Super Sentive Pox-DAB (San Ramon, CA, USA); Biogenex Super Sentive Alk Phos; Consortia HistoCons Pox-DAB (Verona, Italy); Dako ChemMate Pox-DAB (Glostrup, Denmark); Dako ChemMate Alk Phos; Dako CSA Pox-DAB; Dako Envision Pox-DAB; Dako Envision Alk Phos; DBS Universal Pox-DAB (Pleasanton, CA, USA); DBS Universal Alk Phos; DPC CUI Pox-DAB (Fremont, CA, USA); DPC CUI Alk Phos; Lab Vision UltraVision Pox-DAB (Fremont CA, USA); Vector Vectastain Elite Pox-DAB (Burlingame, CA, USA); and Vector Vectastain Elite Alk Phos.

Each antibody was tested at increasing dilutions (10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000 times) starting from the working dilution suggested by the supplier. Every stain was performed three times on different paraffin blocks and in all cases afthe last dilution no appreciable reactivity could be observed. A total number of about 3,000 slides were stained during a period of 2 months.

Results

All detection systems tested, both with peroxidase and alkaline phosphatase, showed a good signal with Ab dilution from 10-50 times respect to the dilution suggested by the supplier.

Three different detection systems were able to visualize all antibodies at a dilution of 1,000 times the suggested one. The same systems were able to show immunoreactivity for CD2O and chromogranin at a 10,000 times dilution.

Table 1. Eight widely used antibodies chosen for immunostaining.

Antibody	Clone	Suppl,er	Antigen retrieval
Vimentin	V9	Dako	PC citrate buffer pH 6 for 2 mm
Estrogen rec.	IDS	Dako	PC citrate buffer pH 6 for 2 mm
Cytocheratin	MNF1 16	Dako	Protease XIV 0.05% 37o for 5 mm
BCL2	124	Dako	PC citrate buffer pH 6 for 2 mm
CD2O	L26	Dako	PC citrate buffer pH 6 for 2 mm
0030	BERH2	Dako	PC EDTA 0.01 M pH 8 for 2 mm
Chromogranin	LK2H1O	Cymbus	PC citrate buffer pH 6 for 2 mm
Ki-67	MIBi	Biomeda	PC citrate buffer pH 6 for 2 mm

PC = pressure cooker