ANTIGEN RETRIEVAL

MARC KEY

Because of the superior preservation of morphology, formalin-fixed paraffin-embedded (FFPE) tissue remains today the medium of choice for most clinical and research studies. However, the loss of immunoreactivity by many antigens caused as a result of fixation in formalin has introduced many challenges. To more fully appreciate the chemical complexity of the fixation of tissue in formalin, the reader is encouraged to consult with two reviews on this topic.[1,2]

The lack of consistency in the use of formalin for fixation between laboratories, especially as it pertains to the variables of concentration, pH and exposure time, has contributed to this complexity as these factors markedly influence the outcome of staining in immunohistochemistry (IHC). Furthermore, the physiological and pathological changes in tissue composition, including the juxtaposition of tissue proteins and their antigenic sites (epitopes), do not allow predicting the outcome of fixation with any degree of certainty. Each antigen may contain from one to many epitopes and each of these may be composed of five or more amino acids. The same may be linked continuously and/or are arranged in three-dimensional proximity as a result of intermolecular folding. Although formalin fixation will allow some epitopes to emerge unchanged (formalin-resistant), others will undergo substantial changes (formalin-sensitive). In this process, cross-linking of unrelated proteins to the target antigen is also possible. The ultimate results are the partial or complete loss of immunoreactivity by the antigen and/or the "masking" of the same.

The first attempt to "improve" the immunoreactivity of formalin-fixed tissue antigens was by use of tryptic digestion prior to immunofluorescent staining.[3] Proteolytic digestion compensates for the impermeable nature of the non-coagulant fixatives by "etching" the tissue and allowing hidden determinants to be exposed. Since then, other proteolytic enzymes including bromelain, chymotrypsin, ficin, pepsin, pronase and various other proteases have been reported for restoring immunoreactivity to tissue antigens with different degrees of success. Use of enzymes may however also entail the risk of destroying some epitopes. Formalin-fixation in conjunction with the digestion procedures need to be optimized and then firmly adhered to.[4]

An entirely new approach for the restoration of immunoreactivity in FFPE tissue sections was reported by Shi et al 1991.[5] This technology used solutions containing various metals and microwave heating for this restoration and the term "antigen retrieval" (AR) was applied for the first time.*

The concept of recovering lost immunoreactivity through exposure to heat near the boiling point of water was at first met with skepticism as it went against the tenet of protecting proteins from the denaturing effect of heat. However, another major step forward in the use of heat was reported by Cattoretti et al[6] who employed a citrate buffer of pH 6.0 instead of the original metal solution for the first successful demonstration in FFPE tissue of the proliferation marker Ki-67. Shortly thereafter, Gown[7] and Leong[8] were able to apply their modifications of AR methods to a wide variety of additional markers. Not only was the staining of many tissue markers improved, but more importantly, a whole new class of antigens, previously found to be non-reactive in FFPE tissue, could be demonstrated successfully for the first time. This included additional proliferation markers, hormone receptors (ER and PR), growth factor receptors (HER2/*neu*), CD markers and others.

More recently, combinations of enzymatic digestion and heat-induced antigen retrieval have been reported. Iczkowski et al[9] combined steam heat with protease digestion, an EDTA buffer of pH 8.0 and obtained staining with monoclonal anti-keratin antibody 34ßE12. This staining was found to be superior to that obtained when only one of these measures was applied. Detailed information can be found in the Product Specification Sheets for IVD approved antibodies.

PRINCIPLE AND TECHNIQUE

The principle of antigen retrieval relies on the application of heat for varying lengths of time to FFPE tissue sections in an aqueous medium. After deparaffinizing and rehydrating the tissue sections, the slides are immersed in an aqueous solution commonly referred to as a "retrieval solution". Although many different chemicals have been proposed, most retrieval solutions share a pH near 2, 6, 8 or 10. Recent systematic comparisons of several retrieval solutions showed that 0.01M TRIS-HCl, pH 1 or 10, was slightly superior to citrate buffer of pH 6.0 and gave the best overall results.[10]

*Alternate terminology used today for "antigen retrieval" includes epitope retrieval, heat-induced epitope retrieval (HIER), target retrieval and target unmasking. The latter two versions have a more generic appeal and have also been applied to the retrieval of nucleic acid targets for in situ hybridization.

Following their immersion in the preheated retrieval solution, the containers holding the slides are exposed to heat. This step is the most critical and the degree to which immunoreactivity can be restored is directly related to the duration of incubation and the attained temperature. The most commonly used heating methods include the use of microwave ovens, autoclaves, steamers, pressure cookers and water baths.[7,8,11-14] Their advantages and disadvantages however are subject to ongoing experimentations whose preliminary results have been summarized by Battifora et al.[15] Although the optimal temperature has not been established, most AR methods apply temperatures near the boiling point of water. The optimal length of exposure to heat may vary from 10 to 60 minutes and depends to some extent on the length of formalin fixation. Twenty minutes appears to be the most satisfactory for most antigens and fixation protocols. Cooling is usually allowed to take place slowly, requiring another 20–30 minutes.

A *Protocol for Antigen Retrieval* (38031) is available upon request. The same has been used with consistently good results at DAKO Corporation for considerable time.

At higher elevations (above 4500 feet or 1200 meters), boiling of the target retrieval solution may occur prior to achieving the desired optimal temperature. In such situations, a recommended alternative procedure is to heat the slides at the maximum achievable temperature and to extend the incubation time of the slides in the target retrieval solution until the desired staining intensity is achieved.[16] An additional possible solution is to use a closed pressure system such as a pressure cooker or autoclave to achieve 95°C. However, each laboratory must determine the best method and target retrieval time for its particular circumstances.

MECHANISM OF ACTION

■ Despite numerous speculative publications regarding the exact mechanism of action by AR procedures, the same remains largely unknown today. In view of the complexity of many different antigens and the largely unknown changes formalin fixation entails, this is not surprising. Heat is obviously of great importance in reversing the damages caused by the fixation with formalin and embedding in paraffin. Whatever the mechanism, some of the cross-linking induced by formalin must remain intact as without the same, proteins would be denatured by the heat used during AR. This seemingly contradictory observation can only be explained by the fact that some cross-links are reversible (Schiff bases), thus restoring the immunochemical integrity of the protein, while others are not (methylene bridges).

Although much remains to be learned, our primary concern is that AR works. Future studies will almost certainly provide insight and help us to understand what we can presently only accept.

CYTOLOGY

■ Methods of AR have also been used successfully for some cytology specimens. It has been shown that by certain modifications, AR procedures can be successfully used for the recovery of estrogen receptor, Ki-67, LCA, HER2/*neu* and cytokeratin. In contrast to FFPE material, the success of this method is not so much related to the mode of fixation as it is readily applicable to aldehyde- and alcohol-based fixatives as well. It was proposed that the immunoreactivity was facilitated by an increase in permeability of cell membranes thus providing access to previously masked cell and nuclear antigens. The modification includes the incorporation into the retrieval solution of a small amount of detergent. It was also necessary to reduce the temperature to 37°C in order to maintain morphology.

TARGET RETRIEVAL FOR IN SITU HYBRIDIZATION

■ Soon after the discovery of AR for immunohistochemistry, investigators applied similar approaches for the recovery of nucleic acid targets in FFPE. Today, many retrieval methods optimized for nucleic acids combine proteolytic digestion with target retrieval. This combined protocol provided better overall results than either method alone.

For greater detail, read the chapter on DNA Probe Technology.

ANTIGEN RETRIEVAL AND ITS USE IN DOUBLESTAINING

■ One of the prerequisites for the successful staining of several antigens in the same tissue section is the removal of all reactants prior to the application of the subsequent primary antibody. This was accomplished by use of an acid elution step, leaving behind only the converted chromogen of the first cycle.

Use of the DAKO EnVision Doublestain System (see Staining Methods chapter) allowed for the staining of two or more tissue antigens separated by the intermittent use of an antigen retrieval reagent instead of the acid elution step. A method for doublestaining based on this procedure is available.

The action of the antigen retrieval reagent is to either physically remove the reactants and/or to alter them sufficiently so they are no longer immunoreactive. This basic method can be extended to accommodate multiple staining within the same tissue specimen provided different chromogens are being employed. The following chromogens (and their colors) were used for simultaneous staining: DAB (brown), Fuchsin (red), Fast Red (red), BCIP/NBT (purple) and nickel-DAB (gray).

CONCLUSION

As immunohistochemical techniques continue to be refined, their application in routine and research pathology is becoming increasingly more useful. Antigen retrieval has made a significant contribution in this endeavor as many markers, previously believed to be lost to the process of FFPE, can now be routinely demonstrated. The benefits are especially obvious with such important diagnostic markers as estrogen and progesterone receptors, Ki-67 and HER2/*neu*. The greater sensitivity in their demonstration gained through AR may however require the reevaluation of the staining results and its clinical interpretation.[10]

As many recent publications have born out, heat-induced AR has been decidedly more successful than the use of proteolytic enzymes and therefore has profoundly affected the practice of immunohistochemistry. However, because of the ongoing proliferation of alternative AR methods, including new and better retrieval solutions for different antigens, some bewilderment exists today among pathologists and histologists. In the future therefore, greater attention will have to be directed to the standardization of fixation in conjunction with, antigen retrieval,[4,10] and very likely optimized for each separate antigen.[17]

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