

PERSPECTIVES

## Antigen Retrieval Techniques: Current Perspectives

Shan-Rong Shi, Richard J. Cote, and Clive R. Taylor

Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, California

**SUMMARY** Development of the antigen retrieval (AR) technique, a simple method of boiling archival paraffin-embedded tissue sections in water to enhance the signal of immunohistochemistry (IHC), was the fruit of pioneering efforts guided by the philosophy of rendering IHC applicable to routine formalin-fixed, paraffin-embedded tissues for wide application of IHC in research and clinical pathology. On the basis of thousands of articles and many reviews, a book has recently been published that summarizes basic principles for practice and further development of the AR technique. Major topics with respect to several critical issues, such as the definition, application, technical principles, and further studies of the AR technique, are highlighted in this article. In particular, a further application of the heat-induced retrieval approach for sufficient extraction of nucleic acids in addition to proteins, and standardization of routine IHC based on the AR technique in terms of a test battery approach, are also addressed. Furthermore, understanding the mechanism of the AR technique may shed light on facilitating the development of molecular morphology.

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**KEY WORDS**

antigen retrieval (AR)  
immunohistochemistry  
ISH  
FISH  
nucleic acid  
formalin

IMMUNOHISTOCHEMISTRY (IHC) has created a wide field for functional (analytical or molecular) morphology, particularly since it has rendered immunoperoxidase methods applicable to routine formalin-fixed, paraffin-embedded tissues based on a series of technical developments. These include increasingly sensitive detection systems and several pretreatments before the immunostaining procedure to recover antigenicity masked by formalin fixation. However, the growing interest of pathologists who attempt further to expand the application of IHC staining on formalin-fixed, paraffin-embedded tissue sections were frustrated by inconsistent results on fixed tissues. More than two decades ago, various alternative fixatives were tried in an attempt to replace formalin in an irreversible chemical reaction of formalin–protein, but they have failed, and it is likely that an ideal fixative will never be found (Larsson 1988). On the other hand, early IHC methods, including initial unmasking

techniques such as enzymatic digestion, failed to yield satisfactory immunostaining for many antigens. Therefore, the search for a simple and effective retrieval method has become a hot topic in IHC since the early 1970s (Taylor and Cote 1994). In response to the need for a more effective method to recover the formalin-modified antigenicity, a high-temperature-heating antigen retrieval (AR) technique, the method of boiling paraffin tissue sections in water, was shown 10 years ago to rendering IHC staining possible on archival formalin-fixed, paraffin-embedded tissue sections (Shi et al. 1991). This AR technique was promptly accepted and employed by pathologists and morphologists worldwide, serving as a simple and effective method to achieve satisfactory immunostaining on archival tissue sections, enthusiastically described as “a revolutionary new technique” and “breakthrough in pathology” (Gown et al. 1993; Boon and Kok 1995).

### Basic Questions: Definition, Application, and Necessary Knowledge for Beginners

In the current literature, the term “antigen retrieval” is predominantly (and originally) defined as a high-temperature heating method to recover the antigenicity of

Correspondence to: Clive R. Taylor, MD, PhD, Dept. of Pathology, University of Southern California Keck School of Medicine, HMR 204, 2011 Zonal Avenue, Los Angeles, CA 90033. E-mail: taylor@pathfinder.hsc.usc.edu

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tissue sections that had been masked by formalin fixation. Several terms were used to represent the same AR heating method, resulting in confusion of the results of literature search by Medline (Taylor and Shi 2000). In contrast, the term "AR" was also applied for non-heating methods, including the enzymatic method. Wide application of the AR technique in pathology and other fields of morphology has demonstrated distinct enhancement of IHC staining on archival formalin-fixed, paraffin-embedded tissue sections for a variety of antibodies in terms of reduction of the detection thresholds of immunostaining (increasing sensitivity) and retrieval of some antigens, such as Ki-67, MIB1, ER-1D5, androgen receptor, and many CD markers, which are otherwise negative in IHC. Therefore, the AR technique plays a role in amplifying the pre-detection phase, in contrast to other approaches of amplification in phases of detection (from multistep to polymeric detection systems) and post-detection (enhanced substrate such as DAB using metal, imidazol, CARD, anti-end product, gold-silver enhancement method) (Shi et al. 2000b,d).

Studies have also revealed that the AR heating method could achieve satisfactory results in IHC for tissues embedded in plastic embedding media used for immunoelectron microscopy (IEM), celloidin-embedded tissues, or non-embedded tissue slices, as well as in cell smear preparations fixed in non-crosslinking fixatives (Boon et al. 2000; Evers and Uylings 2000; Shi et al. 2000c,d; Stirling 2000; Suurmeijer and Boon 2000). The heat-induced retrieval approach has also been applied to in situ hybridization (ISH), TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling], and FISH (fluorescence in situ hybridization) (Bull and Harnden 1999; Kitayama et al. 1999; Gu et al. 2000; Lucassen et al. 2000). For multiple IHC staining, heat was used between each round of the immunostaining procedure to block crossreactivity in addition to retrieval of antigenicity (Lan and Nikolic-Paterson 2000). The AR heating technique was also successfully applied for sufficient extraction of proteins from archival tissue (Ikeda et al. 1998).

Although most publications have reported satisfactory results of AR-IHC, several issues must be kept in mind when this technique is used.

First, not all antigen structures modified by formalin can be restored using conventional AR protocols. Therefore, a "test battery" can be used to establish an optimal AR protocol for certain antigens under investigation (Shi et al. 2000b,d).

For a few proteins, higher temperature (boiling) may induce a negative result of IHC staining. In this case, a lower-temperature heating treatment or a combining retrieval protocol (heat and enzyme digestion or CARD) may provide better results (Cattoretto and

Fei 2000; Elias 2000; Roth et al. 2000). Recent studies have revealed that a combination of the AR heating method with a polymeric labeling two-step detection system could yield a stronger signal of IHC to circumvent the reduced sensitivity due to storage of paraffin sections (Shi et al. 1999b). Zhang et al. (2000) developed an improved IHC method based on the AR technique and two-step detection reagent (EnVision; DAKO, Cambridge, UK) to detect enteroviral antigen in myocardium and other tissues for laboratory diagnosis of enterovirus-associated diseases and for studying the mechanism of virus persistence in chronic myocardial disease.

Knowledge of the exact localization of a certain protein (antigen) in tissue is critical to interpret not only the accuracy of IHC staining results but also the reliability of AR treatment. Many excellent pioneering studies have given examples of approaches to scientific validation of the exact localization of a protein on the basis of morphology and biochemistry or other correlated fields (Sternberger 1978; Baskin and Stahl 1982). For this purpose, a test model system, including a fresh cell line and tissue and a formalin-fixed, paraffin-embedded cell line and tissue that are matched in a comparable fashion, is recommended to form such a basis for scientific knowledge of immunolocalization for certain proteins of interest (Hawes et al. 2000; Shi et al. 2000b). In addition, control groups, including a tissue section that is not treated by AR, are required to rule out any false-positive results or altered immunostaining pattern (Shi et al. 2000d). Many articles recently published in this journal have described sophisticated studies to validate the exact localization of proteins by correlated localization of nucleic acids in cells or tissues (Cataltepe et al. 2000; Sato and Sato 2000). For example, Garcia-Vitoria et al. (2000) demonstrated localization of neuronal nitric oxide synthase (nNOS) in rat gastric epithelium based on comparisons of localization of protein (IHC) and mRNA (ISH) and on polyclonal vs monoclonal antibodies, using the AR heating method for both light and electron microscopy to investigate discrepancies in the literature. Western blotting was also used to confirm nNOS protein localization in gastric epithelium. Their conclusions are convincing.

Although the AR technique is a simple method, it is necessary to understand the factors that influence the effectiveness of IHC staining, particularly two major factors, the heating conditions and the pH value of the AR solution. The heating condition represents heating temperature (T) and the time of heating (t), appearing to be a reverse correlation as  $T \times t$  (i.e., the higher the temperature, the shorter the heating time), to develop an optimal AR protocol after a test battery approach (Chaiwun et al. 2000; Evers and Uylings 2000; Leong and Zaer 2000; Shi et al. 2000d).

## Standardization

The demand for standardization of IHC for quantitation is ever more pressing because of the emergence of a new field of translational research that requires quantitation of the differential expression of various prognostic markers for cancer, based on retrospective study of clinical cases with known clinical outcomes (Pertschuk and Axiotis 2000; Shi et al. 2000b). Nevertheless, standardization of IHC is a huge challenge that will require great effort and more research. The AR technique may contribute to standardization of IHC staining for archival formalin-fixed tissue based on the test battery approach to equalize intensity of IHC staining under variable conditions of fixation and processing (Shi et al. 2000b,d). On the other hand, a satisfactory result of AR-IHC must be evaluated and compared with clinical data and other laboratory studies, such as Western blotting and molecular biological methods, to monitor the optimal AR protocol (Pertschuk and Axiotis 2000). A model system of an antigen matrix model and the above-mentioned matched fresh-fixed tissue model may be helpful in establishment of internal control and external control groups for standardization of IHC and to reach more accurate quantitative IHC (Shi et al. 2000b).

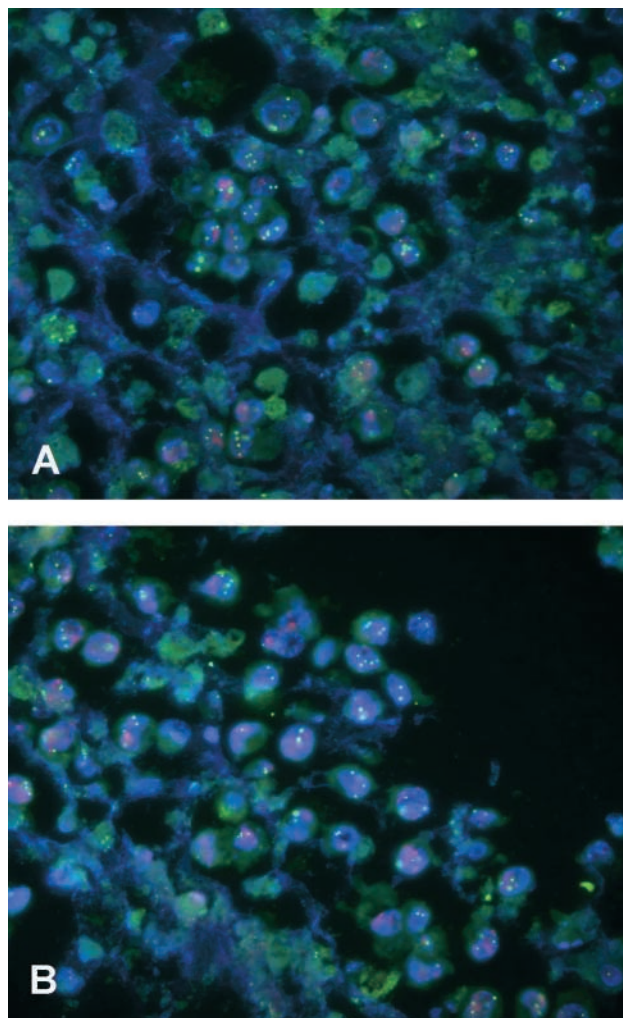
Simplifying the immunostaining procedure is one of the potential strategies for standardization of IHC, and optimal combination of various amplification approaches to achieve a satisfactory result may be based on the principle of simplification of all techniques used (Kawai and Osamura 2000; Merz et al. 2000; Shi et al. 2000b; Taylor et al. 2000).

## Extraction of DNA/RNA by the Heating Retrieval Method

An interesting issue for further development of this heat-induced retrieval approach for macromolecules other than protein was raised by the successful application of the heat retrieval method for enhancement of ISH (Sibony et al. 1995). Oliver et al. (1997) comprehensively compared different pretreatment regimens used to increase the sensitivity of the ISH signal in archival paraffin-embedded tissue sections. The results were evaluated by computer-assisted image analysis, with the conclusion that microwave retrieval regimens may achieve the same sensitivity as that obtained on frozen tissue sections using synthetic oligonucleotides as probes, at the same time retaining satisfactory morphology on archival paraffin-embedded brain tissue. More recently, the high-temperature heating retrieval method has been adopted for FISH to achieve an improved signal, generating signal intensity comparable to that obtained by the regular enzyme digestion method. The heat-induced retrieval method is much easier to control and is a simpler procedure to handle (Bull and

Harnden 1999; Kitayama et al. 1999). In our recent study of Her-2/neu gene amplification in urinary tract cancer, enhanced FISH signals of Her-2/neu have been achieved using a routine heat-induced retrieval protocol (microwave boiling of tissue sections in citrate buffer, pH 6.0, or EDTA-NaOH solution, pH 8.0), with results comparable to those of enzyme digestion (Figure 1).

Can the heat-induced retrieval method be used for sufficient extraction of DNA/RNA from archival paraffin-embedded tissue based on the achievements



**Figure 1.** Comparison of pretreatment protocols for fluorescence in situ hybridization (FISH) using PathVysion HER-2/CEP 17 DNA probe kits (Vysis; Downers Grove, IL) on paraffin sections of a breast cancer cell line that had been fixed in 10% neutral buffered formalin and processed routinely. Red color (rhodamine) indicates HER-2/neu; green color (FITC) indicates the CEP 17. Nuclei were counterstained with DAPI (blue color). **(A)** High-temperature heating was used as the retrieval protocol (microwave heating at boiling in citrate buffer, pH 6.0, for 10 min). **(B)** Pretreatment with protease digestion for 15 min at 37C. A comparable signal was achieved by using the simpler heating method. Original magnification  $\times 1000$ .

of ISH and FISH using the heating method? Extraction of DNA from archival formalin-fixed, paraffin-embedded tissue was accomplished as early as 1985 without the use of the heat-induced retrieval technique (Goelz et al. 1985; Dubeau et al. 1986). After use of PCR to amplify the signal of nucleic acids extracted from archival tissue, heating was primarily based on the initial heating required by the PCR technique as a step to denaturation of DNA (Shibata et al. 1988). The fact that high-temperature heating could be used as an approach to enhance the extraction of nucleic acids or to increase the efficiency of subsequent detection of a target sequence (i.e., heat-induced retrieval for nucleic acid) was not emphasized in the literature until recently. A few articles have discussed this critical issue in a similar manner to the presentation of the AR technique used for IHC (Frank et al. 1996; Masuda et al. 1999; Coombs et al. 1999). Frank et al. (1996) compared five protocols for extracting DNA from archival formalin-fixed, paraffin-embedded tissue sections and found that proteinase K digestion with detergents followed by phenol-chloroform extraction was not more effective than using the simple boiling method (directly immersing the tissue sections in deionized, distilled, autoclaved water without a dewaxing procedure in a microcentrifuge tube and heating in a boiling water bath for 8 min). Masuda et al. (1999) studied the mechanism of chemical modification of synthetic oligo-RNA by formalin to demonstrate and establish a high-temperature heating approach for retrieval of RNA extracted from routinely processed formalin-fixed, paraffin-embedded tissue sections. Their research is an excellent beginning in this field.

Studies of chemical reactions between formaldehyde and nucleic acids also have demonstrated that several basic reactions are similar to those observed in formalin-protein reactions (McGhee and von Hippel 1977a,b). Basic chemical reactions between nucleic acids and formalin indicate that the same steps occur as with formalin-protein reactions. The first step is an "addition reaction." All amino and imino groups of the nucleic acid bases can be replaced with a hydroxymethyl (methylol) group ( $-\text{CH}_2\text{OH}$ ). As with protein, this step is then followed by a condensation process which is, however, a slower reaction that may need more than 1 week to form a stable methylene compound. Adenine appears to be the most critical base in the crosslinking reaction with formalin because it is rich in amino groups. In addition, the rate of the addition reaction of mono-methylol ( $-\text{CH}_2\text{OH}$ ) groups is observed to be much higher for adenine (40%) (Masuda et al. 1999). The chemical reaction of the condensation step is the same kind of reaction found in the formalin-protein crosslinking process. In any case, the methylene bridge ( $-\text{CH}_2-$ ) is the major

structural element of crosslinking, and may be formed in nucleic acid, nucleic acid-protein, and protein-protein interactions (Auerbach et al. 1977). Similar to the formalin-protein reaction, the overall rate of formalin-induced modification of DNA is dependent on both temperature and pH values. As with protein, most reactions of formalin-DNA may be reversible, with the possible exception of the adenine-formaldehyde reaction under certain conditions.

It appears that a subtle merger of the high-temperature heating AR technique for protein antigens with high-temperature heating retrieval of nucleic acids is found in recent literature. Based on the similarity of chemical reactions between protein-formalin and nucleic acid-formalin discussed previously, it is reasonable to "borrow" from the heat-induced retrieval technique of protein to improve extraction of nucleic acids to further speed development of this new technique for molecular morphology.

### Mechanism of AR

A better understanding of the mechanisms of formalin fixation and antigen retrieval may be helpful in the further development of the retrieval technique for enhancement of IHC and ISH, and for more efficient extraction of proteins and DNA/RNA from archival tissue. Although the exact mechanisms of formalin fixation are still unknown, AR-IHC has shed some light on the mechanism of protein-formalin interactions. First of all, the success of AR has shown that the modification of protein structure by formalin is reversible under certain conditions, such as high-temperature heating or strong alkaline treatment. Several hypotheses have been proposed to explain the possible mechanism of AR-IHC, including a recent study of calcium-induced modification of protein conformation conducted by Morgan et al. (1994,1997). These authors suggested that calcium complex formation with proteins in formalin-fixed tissue may mask antigens and that the release of calcium from this cage-like calcium complex may require a considerable amount of energy, such as high-temperature heating and calcium chelation by citrate. Their hypothesis was based on a test showing that exposure of tissue to calcium chloride ( $\text{CaCl}_2$ ) in the AR solution blocked immunostaining of MIB1. Nevertheless, their theory is challenged by subsequent observations showing that calcium-induced modification of protein conformation induces loss of antigenicity for some antigens such as MIB1 and thrombospondin. This calcium-induced loss of IHC staining for certain antigens, which is consistent with biochemical studies in a similar fashion (Dixit et al. 1986), is independent of formalin fixation and does not necessarily imply a role for calcium in AR (Shi et al. 1999a).

We have suggested a possible mechanism of the AR technique, i.e., the loosening or breaking of the crosslinkages induced by formalin fixation (Shi et al. 1991, 1992). We believe that a critical element in the mechanism of AR-IHC may be based on heat- or chemical-induced modification of the three-dimensional structure of "formalinized" protein, restoring the condition of a formalin-modified protein structure back towards its original structure. In other words, the mechanism of AR-IHC appears to involve a re-naturation of the structure of fixed proteins through a series of conformational changes, including the possible breaking (hydrolysis) of formalin-induced crosslinkages, the entire process being driven by thermal energy from the heat source (Shi et al. 1997, 2000a).

The hypothesis of the heat-induced re-naturation is based on an essential principle of immunology, i.e., antigen-antibody recognition is dependent on protein structure. Antibodies recognize specific epitopes localized in a particular spatial configuration within the protein molecule. This is particularly true for discontinuous antigenic determinants, which are composed of residues from different parts of the amino acid sequence. A conformational change in a protein caused by formalin fixation may mask the epitope and thus affect the antigenicity of proteins in formalin-treated tissue. The AR method may lead to a re-naturation or at least partial restoration of the protein structure (induced by high-temperature heating or other non-heating procedures), with re-establishment of the three-dimensional protein structure to something approaching its native condition.

The exact chemical reaction involved in this modification-re-naturation mechanism is not clear. The most likely process that would restore part of the native configuration of a formalin-modified antigen is the hydrolysis of crosslinkages and other formalin adducts that result from formalin-protein fixation. In support of this hypothesis, evidence in the literature has shown that extending the period of washing formalin-fixed tissue in water may reverse the loss of immunoreactivity (Pearse 1968). Further studies concerning the mechanism of AR-IHC should focus on studying the alterations of protein structure that take place during fixation and "unfixation" or retrieval. Recent studies using the AR technique for IHC staining also support the hypothesis that hydrolysis is a critical factor in this restoration (re-naturation) of formalin-modified protein conformation. For example, a negative immunostaining result may be observed if archival paraffin-embedded tissue sections are heated in pure glycerin above 100°C. However, adding a 10% water solution to this pure glycerin leads to a satisfactory positive immunostaining result for the same section (Beebe 1999).

Biddolph and Jones (1999) demonstrated that heat-

ing is the most important factor for the AR technique to achieve a satisfactory result with IHC. They found that incubation of archival paraffin-embedded tissue sections in all AR solutions, including the citrate buffer solution of pH 6.0 at room temperature or at 37°C for 8–72 hours, failed to achieve satisfactory positive staining for a variety of antibodies tested. However, this does not preclude the possibility that immersion of archival tissue section in water or buffer solution at room temperature for a much longer time (months or years) may achieve a positive result. Such an outcome is consistent with our working principle regarding the overall heating conditions (heating temperature times time of incubation) as previously described. A much lower temperature theoretically requires a much longer period of incubation (Shi et al. 1996, 1997; Taylor et al. 1996). The fact that the pH value of the AR solution is another important factor influencing the result of AR-IHC may also support the effectiveness of hydrolysis as a basic mechanism of AR because hydrolysis of protein is pH-dependent and formalin-induced modification of nucleic acid and protein structures is also pH-dependent.

In conclusion, the heat-induced AR technique has achieved wide application in pathology as well as other fields of morphology, and has provided a potential approach to standardization of routine IHC for approaching quantification of IHC, as well as more effective extraction of nucleic acids from archival paraffin-embedded tissues. Further studies of this AR technique, including the mechanism of AR, may shed new light in molecular morphology.

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