Diagnostic Immunohistochemistry

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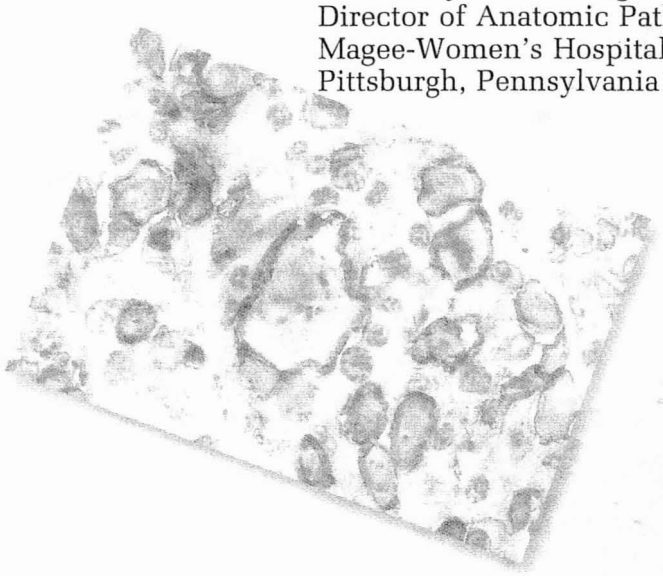

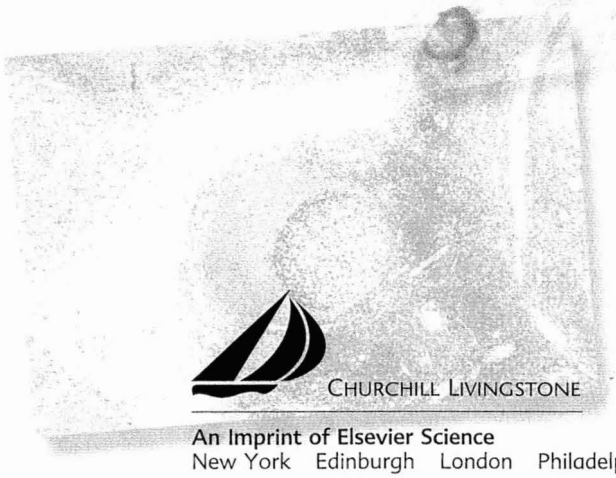

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ence that "storage-induced" decreases in IHC staining are relatively uncommon, and most of these adverse effects can be recovered by AR treatment. For example, combination of the AR treatment and use of a sensitive polymeric labeling two-step detection system achieves satisfactory results for antibodies to p53 (Pab-1801), $p21^{WAF1}$ (Ab-1, Oncogene) and $p27^{Kip1}$ (DCS-72.F6, NeoMarkers),[17, 91] even after prolonged storage.

The AR technique has utility not only for enhancement of IHC staining on archival tissue sections but also for standardization of routine IHC.[17, 38, 39, 91] A key element in the appropriate use and standardization of the AR technique for IHC is understanding major factors that influence the effectiveness of AR.

In general, major factors that influence the quality of results of AR-IHC include heating temperature and heating time (heating condition T × t) and the pH value of the AR solution. The chemical components and molarity of the AR solution are cofactors that may influence the effectiveness of AR-IHC in certain instances.

Heating Conditions. As noted previously, the heating AR-IHC method is based on biochemical studies of Fraenkel-Conrat and coworkers, who documented that the chemical reactions that occur between protein and formalin may be reversed, at least in part, by high temperature heating or strong alkaline hydrolysis.[31–33] We demonstrated that the use of conventional heating at 100°C may achieve results similar to those obtained by microwave heating and also that distilled water could be used as the AR solution, albeit with slightly less effect.[34] Subsequently, several publications reported similar results by using conventional heating.[38, 39, 125, 126] Malmstrom and colleagues[122] performed AR-IHC on proliferating cell nuclear antigen (PCNA) (PC10 and 19F4) using distilled water as the AR solution on routinely processed paraffin sections of urinary bladder carcinoma and obtained good results.

The chemical reactions occurring during the formalin fixation process remain obscure. Mason and O'Leary[124] demonstrated that the process of cross-linking does not result in discernible alteration of protein secondary structure, at least as determined by calorimetric and infrared spectroscopic investigation. They also noted that significant denaturation of unfixed purified proteins occurred at temperature ranges from 70°C to 90°C, whereas similar temperatures had virtually no adverse effect on formalin-fixed proteins (i.e., formalin-fixed proteins are more heat-stable). Thus, the AR heating technique, using high temperature heating of tissue sections fixed in formalin, may take advantage of the fact that the cross-linkage of protein produced by formalin fixation may "protect" the primary and secondary structure of formalin-modified protein from denaturation during the heating phase, while allowing some reduction of cross-linkages at the surface of the molecule, thereby restoring antigenicity. Although the mechanism of action of the AR technique is not clear, it appears unlikely, based on the preceding observation that "protein denaturation alone is the mechanism" as advocated by Cattoretti and colleagues.[43]

In general, the heating conditions appear to be the most important factor in the effectiveness of AR.[37–41, 54–56, 125–130] The evidence may be summarized as follows:

- Significant enhancement of immunohistochemical staining can be achieved by using high-temperature heating of routinely processed paraffin-embedded tissue sections in pure distilled water.[34, 125, 131–133]
- Higher temperatures in general yield superior results.[38, 39, 55–57, 125, 128] An optimal result for AR-IHC is correlated with the product of the heating temperature and the time of AR heating treatment (T [temperature of heating AR procedure] × t [period of heating time]).[110, 129]
- An equivalent intensity of AR-IHC can be obtained using different buffers as AR solutions if the pH value of AR solutions are monitored in a comparable manner, thereby demonstrating that individual specific chemical constituents are not necessary factors in yielding a satisfactory result.[47–49]
- Our early experience that even prolonged exposure of paraffin sections in citrate buffer solution (or indeed any buffer) without heating gave no noticeable AR effect has subsequently been confirmed by numerous studies.[52, 54, 134]

pH and Chemical Composition of the Antigen Retrieval Solution. The pH value of the AR solution is important for some antigens. From a 1995 comparative study,[49, 50] we concluded that antigens fell into three broad categories with respect to the importance of pH on AR:

1. Most antigens showed no significant variation using AR solutions with pH values ranging from 1.0 to 10.0.
2. Certain other antigens, especially nuclear antigens (e.g., MIB1, ER) showed a dramatic decrease in the intensity of the AR-IHC at middle-range pH values, but optimal results at low pH.
3. A small group of antigens (MT1, HMB-45) showed negative or very weak focally positive immunostaining with a low pH (1.0 to 2.0) but excellent results in the high-pH range (Figs. 1–17 and 1–18).

Evers and Uylings[127] also found that the AR-IHC is pH- and temperature-dependent. They tested two antibodies, MAP-2 and SMI-32, and indicated that the optimal pH values were pH 4.5 for MAP-2, and pH 2.5 for SMI-32. They also concluded that it is not important what kind of solution is used as long as the pH is at an appropriate level.

In conclusion, major factors that influence the results of AR IHC stain include heating temperature and heating time (heating condition T × t) and the pH value of the AR solution. The chemical composition and molarity of the AR solution are cofactors that may influence the effectiveness of

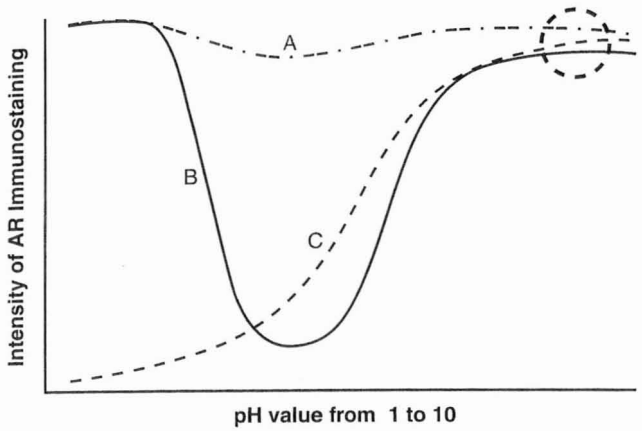

Figure 1–18. Schematic diagram of the three patterns of pH-influenced AR immunostaining. Line A (pattern of type A) shows a stable pattern of staining with only a slight decrease in staining intensity between pH 3 and pH 6. Line B (pattern of type B) shows a dramatic decrease in staining intensity between pH 3 and pH 6. Line C (pattern of type C) exhibits an ascending intensity of AR immunostaining that correlated with increasing pH values of the AR solution. Circle *(right)* indicates the advantage of using an AR solution of higher pH value. (Reproduced, with permission, from Shi S-R, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. Journal of Histochemistry & Cytochemistry 43:193–201, 1995.)

tissue sections. Titration (dilutions) may also differ.

Because the success of the IHC staining method depends on the correct application of both histologic and immunologic techniques, it is recommended that the user be familiar with the literature concerning the antigen under investigation before performing IHC staining. In particular, it is important to know the following:

- The cellular localization of the antigen base
- The specificity of the primary antibody
- The results of previous IHC staining tests (from the literature, and especially from the experience of the performing laboratory), with respect to any adverse influence on the antigen from tissue fixation processing, and the value of any pretreatment procedure such as heat-induced AR

In addition, detailed information regarding the reagents, particularly the primary antibody and detection system—such as the manufacturer, clone number of monoclonal antibody, and recommended concentration—is helpful in achieving a successful result.

Antigen Retrieval Protocol

Microwave Heating Method

(Shi et al., 1991)[34]

1. Deparaffinized slides are placed in plastic Coplin jars containing AR solution; it is recommended that the same number of slides be used every time, using "blanks if necessary" to ensure consistent heating.
2. Jars are covered with loose-fitting screw caps and heated in the microwave oven for 10 minutes. The 10-minute heating time is divided into two 5-minute cycles with an interval of 1 minute between cycles to check the fluid level in the jars. If necessary, more AR solution is added after the first 5 minutes to avoid drying out the tissue sections. It is recommended that the heating time be standardized by beginning to count the time only after the solution has reached boiling in order to avoid discrepancies among laboratories when using various microwave ovens.
3. After completion of the heating phase, the Coplin jars are removed from the oven and allowed to cool for 15 minutes.
4. Slides are then rinsed twice in distilled water and in phosphate-buffered saline (PBS) for 5 minutes and are ready for IHC staining.

Various heating methods, including conventional heating in water bath, pressure cooker, steamer, and autoclave, may be used and may achieve results similar to those with IHC. Our studies have demonstrated that different heating methods can

Table 1–5. Test Battery Suggested for Screening an Optimal Antigen Retrieval Protocol

TRIS-HCl Buffer	pH 1 to 2	pH 7 to 8	pH 10 to 11
Super high, 120°C*	Slide No. 1	Slide No. 4	Slide No. 7
High, 100°C for 10 minutes	Slide No. 2	Slide No. 5	Slide No. 8
Mid-high, 90°C for 10 minutes†	Slide No. 3	Slide No. 6	Slide No. 9

* The temperature of super high at 120°C may be reached by either autoclaving or microwave heating with longer time.
† The temperature of mid-high at 90°C may be obtained by either a water bath or a microwave oven monitored with a thermometer.
Note: One more slide may be used for control without AR treatment. The citrate buffer pH 6.0 may be used to replace TRIS-HCl buffer pH 7 to 8, as the results are the same.
Adapted from Shi, S-R, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: Past, present, and future. J Histochem Cytochem 1997;45:327–343.

tives had no advantage over formalin fixation when used with the labeled streptavidin method.[151]

Battifora and coworkers did a comparative analysis between DCC and IHC in 166 patients with 6-year follow-up and concluded that there was 94% agreement between the methods when the DCC threshold of 20 fmol/mg was used with the H222 antibody.[152] Several studies subsequently confirmed that the results obtained with the H222 antibody gave results comparable to results from frozen section studies.[148, 153–159]

The appearance of second-generation ER antibodies (ER1D5, DAKO, Carpinteria, CA) combined with newer antigen retrieval methods[160–162] has rendered the DCC method obsolete.[163]

Early concordance studies with ER ICA and the DCC method showed good correlation and high sensitivity and specificity in terms of correlation with H222 results,[164] overall survival, or disease-free survival.[165–169]

Quantitation of results of the ICA is an issue of some controversy. A survey of the literature reveals that some authors set a "positive" ER result at greater than or equal to 5% nuclear staining, whereas others set a "positive" result at a minimum of 10% nuclear staining. Still others rely on the "H score," which includes measures of percent positive nuclei with intensity of nuclear staining.

Pertschuk and colleagues and Taylor and associates argued that using a percentage of nuclear staining of 10% as a minimum for a positive result was reproducible and correlated well clinically.[167, 170, 171] Ferno and associates also found correlation with clinical response with the value of 10% nuclear staining, finding no additional value in the nuclear staining intensity.[172]

Schultz and coworkers and Remmele and associates compared visual subjective immunoscoring with image analysis; both groups concluded that image analysis quantitation was no better than visual semiquantitation, which is cheaper and simple and rapid.[173, 174]

In a review of the topic, Barnes and colleagues used a triad of staining intensity, percent positive cells, and degree of heterogeneity of staining to arrive at an index number that is predictive of endocrine response.[175] These investigators set their cutoff points for a positive result based on the results of DCC-associated endocrine response, with a sensitivity of 71% and a specificity of 62%.[175]

Clark and associates concluded that even patients with 1% positive staining for ERs/PRs benefited with adjuvant endocrine treatment.[176]

Reproducibility studies of scoring the ICA for ERs/PRs are few. van Diest and associates observed that scoring at the extremes (zero versus strongly positive) gave the best reproducibility, whereas intermediate scores were more difficult to agree on, yielding an overall agreement of 61%.[177]

A high concordance rate has been reported between measurements made by the DCC and by IHC on formalin-fixed, paraffin-embedded tissues.[178, 179]

Quantitative results of the ICC method correlate closely with biochemical results and are predictive of prognosis.[180–184] Few studies have examined whether the presence of ER predicts an endocrine response, and of the studies that have, the numbers of patients in the study have been small.[185] Veronese and colleagues, in a study using ER1D5, found that ER1D5 staining was predictive of response to tamoxifen in 65 homogeneously treated patients, and was a discriminator for relapse-free and overall survival.[186] Barnes and coworkers and Goulding and colleagues confirmed that the ER by IHC correlated better than the DCC method, and the results were strongly related to patient outcome, regardless of the method of immunoscoring.[187, 188]

In one of the largest studies with long follow-up, Harvey and associates studied the correlation with DCC and the response to endocrine therapy in 1982 patients and found that the ER IHC assay correlated well with DCC, predicted therapeutic response groups, and had distinct advantages over the DCC method.

All the controversy regarding the interpretation of what constitutes positive ER by IHC has been laid to rest by a statement issued in the November 1–3, 2000 National Institutes of Health (NIH) Consensus Statement on Adjuvant Therapy for Breast Cancer: Any positive nuclear ER immunostaining is considered to be a positive result and should be a definitive reason for instituting antiestrogen therapy for a patient.

The fact that ER/PR by IHC can be performed on minute quantities of tissue is a distinct advantage, especially for patients with a diagnosis of carcinoma made by FNA.[182] Several studies demonstrate the high sensitivity and specificity and correlation with DCC for ER/PR determination in FNA specimens.[131, 189]

The ER/PR IHC methods can also be applied to ThinPrep techniques, and they work well in the proprietary fixative PreservCyt (Cytyc, Boxborough, MA).[190, 191]

Rosen was the first to describe tumor histopathologic correlations with ER/PR expression.[192] Tumor types that tend to be ER+ include tubular, mucinous, and papillary carcinomas, along with ductal carcinomas of good (low) nuclear grade.[140, 141]

Progesterone nuclear staining by the IHC method is more heterogeneous than ER,[193] and may be a cause of false-negative results, especially in core biopsies[135] or needle aspirates. My institution has seen similar results on some core biopsies; as a result, if we obtain a negative PR or ER result on a core biopsy specimen, we repeat the test on the excisional breast lumpectomy specimen.

The sensitivity, specificity, and concordance studies for PR using antibody 1A6 have all been comparable to the study results of ER.[194–197]

Much remains to be learned about the biology of sex steroid receptors and their significance in the management of patients with breast cancer. The discovery of an additional form of ER (ER