PART I

RECENT ADVANCES IN ANTIGEN RETRIEVAL TECHNIQUES AND ITS APPLICATION

CHAPTER 1

STANDARDIZATION OF ANTIGEN RETRIEVAL TECHNIQUES BASED ON THE TEST BATTERY APPROACH

SHAN-RONG SHI and CLIVE R. TAYLOR

Following the development of the antigen retrieval (AR) technique in 1991,[1] hundreds of articles have been published worldwide that document its application in immunohistochemistry (IHC) for archival formalin-fixed, paraffin-embedded (FFPE) tissue sections. In addition, there are numerous articles that focus on standardization of the AR technique, stimulated by the current demand for a more quantitative method of IHC.[2–6] The critical importance of standardization of antigen retrieval immunohistochemistry (AR-IHC) has been emphasized by the American Society of Clinical Oncology and the College of American Pathologists in their Guideline Recommendations for human epidermal growth factor receptor 2 (HER2) testing in breast cancer.[7] The problem was, however, recognized and addressed to some degree much earlier. To optimize the results of AR-IHC in formalin paraffin sections, a "test battery" approach was proposed in 1996.[8] The basic principle of this approach is based on the fact that two major factors influence the achievement of a satisfactory result of AR-IHC, namely, the heating condition (heating temperature × heating time) and the pH value of the AR solution (in which the FFPE tissue sections are immersed during heating).[8–12] In practice, it suffices to test the (new) primary antibody using three different pH values, ranging from low (acidic), moderate (neutral), and high (basic) buffer solutions (or other comparable commercial AR solutions) under three heating temperatures: low (below boiling), moderate (boiling), and high (pressure cooker or autoclave), to establish an optimal AR protocol for tested antibodies (Table 1.1). Subsequently, numerous investigators have demonstrated the advantages of using this simple test battery method. As emphasized by O'Leary,[2] the use of a "test battery" provides a rapid way to optimize AR for a particular antibody/antigen pair.

Antigen Retrieval Immunohistochemistry Based Research and Diagnostics,
Edited by Shan-Rong Shi and Clive R. Taylor

TABLE 1.1 Test Battery Suggested for Screening an Optimal Antigen Retrieval Protocol

	Tris–HCl Buffer		
	1.0–2.0	7.0–8.0	10.0–11.0
	(Slide #)[a]	(Slide #)[a]	(Slide #)[a]
Super-high (120°C)[b]	1	4	7
High (100°C), 10 min	2	5	8
Mid-high (90°C), 10 min[c]	3	6	9

[a] One more slide may be used for control without AR treatment. Citrate buffer of pH 6.0 may be used to replace Tris–HCl buffer, pH 7.0–8.0, as the results are the same.
[b] The temperature of super-high at 120°C may be reached by either autoclaving or microwave heating at a longer time.
[c] The temperature of mid-high at 90°C may be obtained by either a water bath or a microwave oven monitored with a thermometer.

Reprinted with permission from Shi et al., *J. Histochem. Cytochem.* 1997; 45: 327–343.

Recent studies have further extended the application of this approach to establish and validate the optimal AR protocol for various antibodies (exemplified in Table 1.2) with different detection systems, employing a multi-tissue microarray (TMA) to achieve a rapid and accurate evaluation.[26,27] It has become apparent that significant differences can be found in IHC staining results among various primary antibodies and different detection systems with the use of different AR protocols. For example, Pan et al.[27] evaluated the consistency of IHC staining for four antibodies to thyroid transcription factor (TTF)-1, manufactured by Dako, Zymed, Novocastra, and Santa Cruz, employing TMA blocks of 77 hepatocellular carcinomas and 334 nonhepatic epithelial tumors, using two solutions for AR treatment. Significantly different cytoplasmic IHC staining results were observed among different antibodies, as well as different AR solutions (e.g., Dako Target Retrieval Solution vs. ethylenediaminetetraacetic acid [EDTA] buffer at pH 8.0). In another study, Gill et al.[21] standardized an AR method for IHC staining using antibody to a neuronal nuclear protein, NeuN, as the outcome measure. They compared three different pH values of AR solutions including low, middle, and high pH, with heating at three temperatures of 95, 100, or 105°C, for 15 or 20 min. They found that heating FFPE tissue sections in an alkaline pH buffer at high temperature gave the best results. The utility of the test battery approach used to establish optimal AR protocols has been demonstrated by abundant literature as summarized in Table 1.2.

The increasing attention directed to the adverse effects of variation in sample preparation upon the quality of IHC staining of FFPE tissues has served to reinforce the importance of determining the optimal AR method for each antibody/detection system/antigen to achieve optimal retrieval and optimal staining of tissues that may have been processed and stored in different and unknown ways (see Chapter 5 for details). Practically, in considering

TABLE 1.2 Randomly Selected Examples of Test Battery Approach Documented in Abundant Literature

Reference	Sample	Purpose and AR Method	Conclusion
Shi et al.[8]	FFPE tissues of normal spleen, small cell lung ca. bladder ca. with comparable frozen tissues of bladder ca.	To establish an optimal AR protocol for poly- and monoclonal antibodies to retinoblastoma protein (pRB). Tris buffer at three pH values of 1, 6, and 10, heating at autoclave 120°C, MW 100°C, and 90°C for 10 min	An optimal AR protocol of boiling FFPE tissue sections in low pH (1–2) buffer for 10 min was established to achieve a maximal retrieval result.
Ferrier et al.[13]	FFPE tissues of several tumor specimens with matched frozen tissues as comparison	To validate AR-IHC staining protocols for plasminogen activation system testing citrate buffer of pH 2.5, 4.5, and 6.0, 3 M urea, and Tris–HCl of pH 10.0, with MW heating at 97°C for 10–20 min	A pretest based on three different pH value (low, middle, and high) as a test battery is helpful to determine an optimal AR protocol.
Rocken and Roessner[14]	Aldehyde-fixed and Epon-embedded autopsy tissues	To establish an optimal AR protocol for post-embedding IEM of amyloid detection, testing water, citrate buffer of pH 6.0, EDTA of pH 8 as AR solution heating at 91°C, 30 min, and combining etching	Application of test battery proved valuable in assessing appropriate AR protocol.
Shi et al.[15]	FFPE tissues of bladder ca. and cell lines	To establish an optimal AR protocol for a polyclonal antibody to COX-2 (PG-27) using above-mentioned test battery approach	A reduced temperature AR protocol was established.

TABLE 1.2 ***Continued***

Reference	Sample	Purpose and AR Method	Conclusion
Yano et al.[16]	Tissues of insulinoma fixed in 2% glutaraldehyde, postfixed in 1% OsO_4, embedded in Epon	To establish an optimal AR protocol for detection of chromogranin A in ultrathin sections, testing three AR solutions of citrate buffer pH 6.0, EDTA buffer pH 8.0, alkaline solution pH 10.	Considerably improved efficiency of IHC was achieved by MW heating in pH 10 solution with IHC staining at 60°C.
Saito et al.[17]	Aldehyde-fixed cultured *Helicobacter pylori*, embedded in Lowicryl K4M	Using the cultured bacteria as a model to establish optimal AR protocol for post-embedding IEM, based on comparison of heating conditions and various AR solutions: water, phosphate buffer pH 7.4, EDTA pH 7.2, Tris pH 10.0, urea pH 7.2, citric acid pH 6.0, commercial fluid pH 6.0, with heating at 121°C, 99°C, or 65°C	AR in Tris buffer solution of pH 10 showed better IHC staining results for ultrathin sections. AR method should be applied for routine use for post-embedding IEM.
Naito et al.[18]	FFPE tissues of Alport's syndrome and normal portion from resected renal tumor	To establish optimal heating conditions for AR-IHC of mAb to α chains of collagen IV, testing autoclave heating at 105, 110, 115, 121, or 127°C for 6 min, or 127°C for 8 min with buffers of pH 3.5, 6, and 7.4	Heating at two or three different temperatures could be helpful for diagnosis; AR method extends the IHC diagnosis for Alport's syndrome.

TABLE 1.2 ***Continued***

Reference	Sample	Purpose and AR Method	Conclusion
Kim et al.[19]	Archival FFPE tissues of pathology	To investigate optimal AR protocols for 29 antibodies commonly used in pathology, testing 7 different buffers with variable pH value ranging from 2 to 9 under 2 heating conditions	Borate pH 8.0 or Tris pH 9.5 buffer combining with pressure-cooking heating method yielded the best results.
Choi et al.[20]	FFPE tissues of invasive aspergillosis from 16 pediatric cases, fixed in formalin for 6–72 h	To establish an optimal AR protocol for mAb WF-AF-1 (Dako), testing three different retrieval solutions of pH 6.0, 8.0, and 10.0 with MW heating for 10 min	Satisfactory IHC results are achieved using AR with high pH.
Gill et al.[21]	Archival FFPE spinal cord tissue; both paraformaldehyde-fixed frozen rat spinal cord tissue and paraffin-embedded same tissue	To establish an optimal protocol for detection of low-abundance protein (NeuN) in human spinal cord FFPE tissue sections, testing three AR solutions of pH 6, alkaline, and acidic buffer, with three heating conditions: 95, 100, and 105°C	Heating FFPE tissue sections in an alkaline buffer yields most effective AR-IHC staining results.
Du et al.[22]	FFPE tissues of prostate ca., benign prostate hyperplasia, and breast disease	To find optimal AR protocols for IHC staining of p504s, p63, CD10, and Ki-67, testing citrate buffer pH 6.0, EDTA buffer pH 8.0, and 9.0 with MW heating at 700 W for 12, 20, 25, 30 min	Different antigens require variable AR protocols. In general, most antibodies tested showed better results for pH 9.0.

TABLE 1.2 ***Continued***

Reference	Sample	Purpose and AR Method	Conclusion
Luo et al.[23]	Archival FFPE tissues of normal or tumors	To establish optimal AR protocols for 30 commonly used antibodies, testing 9 AR protocols	Different antigens require variable AR protocols to reach the best IHC staining results.
Ge et al.[24]	Murine pancreas and other organs fixed in 10% neutral buffered formalin (NBF) for 6–24 h, embedded in paraffin	Searching for an AR protocol that works with a variety of tissues and antigens, testing AR solutions of Vector buffer pH 6, Tris buffer pH 7.5 (+0.1% Tween-20) with low-and high-power MW heating	Low-power heating AR protocol provides a successful IHC detection for several key antigens in the pancreas.
Slater and Murphy[25]	FFPE prostate cancer and benign tissue sections from pathology	To establish optimal AR protocol for studying relationship of IL-6 and growth hormone, testing three AR solutions of pH 10.0, 7.0, and 2.0, with four heating temperatures of 100, 90, 80, and 70°C	No positive IHC results using AR solutions of pH 7 or 10, but good result was obtained at pH 2, with heating at 80°C for 50 min.
Lyck et al.[26]	Two tissue arrays of predominantly aldehyde-fixed, paraffin-embedded brain tissues, fixed in variable times ranging from 1 day to 10 years	To identify antibodies and protocols that could visualize neurons and glia for quantitative studies, testing 29 antibodies, 4 AR buffers: Tris–EGTA pH 9.0, citrate buffer pH 6.0, and 2 commercial solutions with several heating conditions of MW heating	Application of IHC for quantitative studies of human brain tissue is possible with careful selection of staining method in well-preserved specimens.

Note: All tissue samples are human source unless otherwise noticed.
Ca., carcinoma; MW, microwave.

the busy workload in a clinical service laboratory, we recommend a two-step procedure based on the typical design of a test battery (Table 1.1): in the first step, test three AR solutions at different pH values under one heating condition (100°C for 10 min) to find the optimal pH value; in the second step, test optimal heating conditions based on the optimal pH identified in step 1.[28] Similarly, Hsi[29] recommended using microwave pressure cooker as the standard heating condition for testing two commonly used AR solutions, citrate buffer of pH 6.0 and EDTA solution at pH 8.0, along with protease digestion. With the goal of identifying the optimal AR protocol for a new primary antibody, they used five different concentrations of the antibody, including the manufacturer's recommended dilution, plus two serial twofold dilutions above and below this concentration. As seen in Table 1.2, many investigators have already accepted the basic principle of test battery, incorporating three levels of pH values and three heating conditions (Table 1.1). However, within this model, different investigators have used different heating methods and different AR methods to achieve optimal results for their individual laboratories. With this broad variety of approaches, clearly, we are a long way from achieving a universal method, even if such is possible.

1.1 SEARCHING FOR NOVEL CHEMICAL SOLUTIONS

Namimatsu et al.[30] reported a novel AR solution containing 0.05% citraconic anhydride, pH 7.4, for heating FFPE tissue sections at 98°C for 45 min. They compared the IHC staining results using 62 commonly used antibodies and other conventional AR protocols (0.01 M citrate buffer, pH 6.0 in a pressure cooker; or 0.1 M Tris–HCl buffer containing 5% urea, pH 9.0 microwave heating for 10 min), and found that most antibodies showed stronger intensity with the new method. In particular, some difficult-to-detect antigens such as CD4, cyclin D1, granzyme β, bcl-6, and CD25 gave distinct IHC staining signals only by using the new protocol, leading to a claim that the method might be a candidate for the "universal" approach.

We therefore tested Namimatsu's protocol and also obtained satisfactory results.[31] Among 30 antibodies tested, more than half (53%) showed a stronger intensity of IHC when using the citraconic anhydride for AR, as compared to citric acid buffer, whereas 12 antibodies (43%) gave equivalent results. There was only one antibody (OC-125) that, in our hands, gave a stronger intensity using conventional citric buffer for AR. When using citraconic anhydride for AR, the heating conditions of boiling (100°C) or less than boiling (98°C) temperature yielded identical results for most antibodies tested (90%). However, 3 of 30 antibodies showed lower intensity at 100°C. In addition, some antibodies showed nonspecific background staining at 100°C. In particular, we demonstrated that when using antibody to retinoblastoma protein (pRB), the new protocol had advantages over a previously published low pH

TABLE 1.3 Comparison of pRB-IHC between Frozen and Paraffin Sections Using Four Protocols of AR

Sample	Frozen Section	FFPE Section with Antigen Retrieval			
		Acetic Buffer pH 1–2, 100°C	Citroconic Anhydride 100°C	Citroconic Anhydride 98°C	Citrate Buffer pH 6.0, 100°C
T24	+++	+++, >50%	+++, >50%	+++, >50%	+++, >50%
J82	+	+, >10%	+, >10%	+, >10%	±, <10%
Case 1	—	—	—	—	—
Case 2	Nuclear +++, >50%	+++, >50%	+++, >50%	+++, >50%	++, <50%
Case 3	Perinuclear++, >50%	+, >50%	++, >50%	++, >50%	+, <50%[a]
Case 4	Nuclear +++, >50%	++, >50%	+++, >50%	+++, >50%	++, <50%

Notes: T24 and J82 are cell lines of bladder cancer. Cases 1 to 4 are specimens of human bladder cancer.

[a]Although peripheral area of the slide showed a percentage of positive staining about 50%, the central area of the slide showed significantly weak positive result.

Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309.

protocol,[8] including superior morphologic preservation, greater reproducibility, and more intense staining signal.

As a further motivation, there is evidence that establishing the optimal AR protocol will also contribute to standardization of IHC, through "equalizing" variable IHC staining results obtained following different times of formalin fixation. In the light of the studies described above, further studies were conducted as to the utility of the citraconic anhydride method.

First Step: A comparative study of IHC staining for pRB was carried out using paired sections of frozen versus FFPE cell/tissue samples, comparing citraconic anhydride as the AR solution under two different temperatures (98°C vs. 100°C), with solutions of low pH buffer (acetate buffer, pH 1–2) and citrate buffer (pH 6.0). Findings are summarized in Table 1.3. Conventional citrate buffer yielded inconsistent and weaker signals for all specimens, except the cell line T24 (Table 1.3, Fig. 1.1). Stronger intensity was found in pRB-positive cases, while using the citraconic anhydride for AR (Fig. 1.1), although more nonspecific background staining was observed using citraconic anhydride under boiling condition (Fig. 1.1, C vs. D, and R vs. S).

Second Step: For further evaluation, a comparative IHC study was performed using citraconic anhydride and conventional AR protocols with a TMA of 31 cases of bladder cancer. Findings are summarized in Table 1.4. Only 27 cases were available for evaluation due to loss of tissue cores for four cases. Among 27 cases, there were 6, 8, and 13 cases for strong, moderate

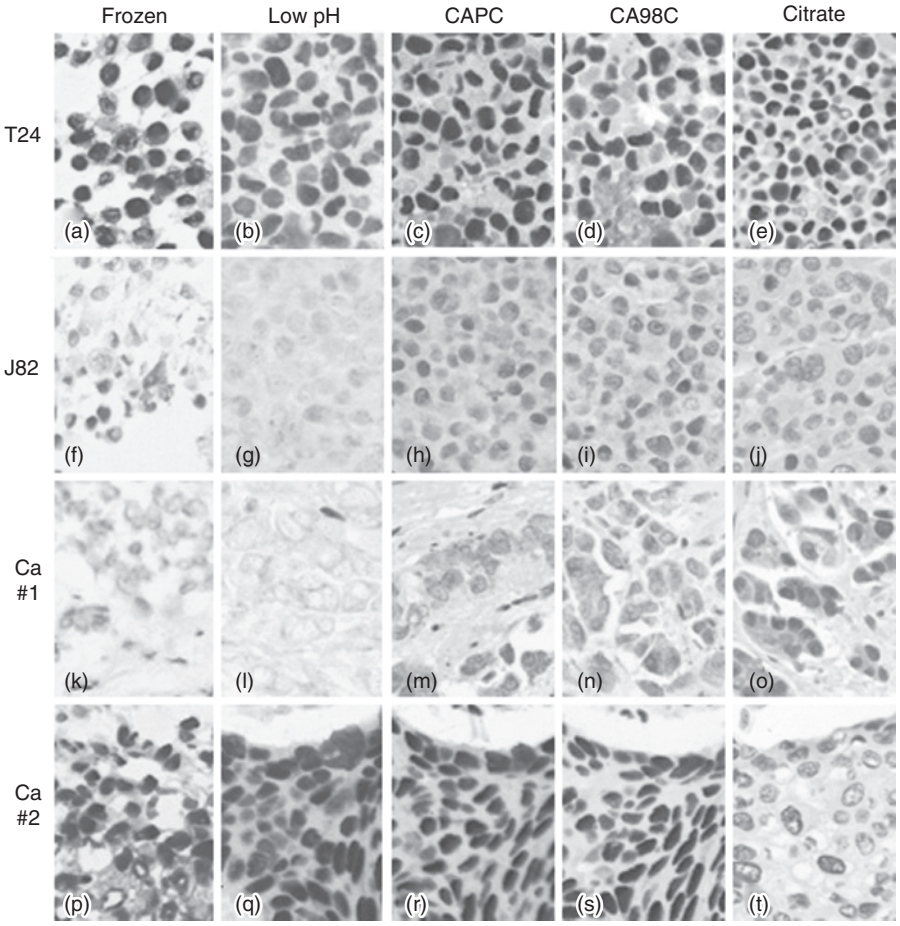

Figure 1.1 Comparison of pRB-IHC staining results for frozen and FFPE tissue sections using four AR protocols. All images are arranged in the same order as given in Table 1.3, indicating all scores indicated in the table. T24 and J82 are two cell lines, Ca #1 and Ca #2 are specimens of human bladder cancer, frozen means frozen cells or tissues fixed in acetone, other terms listed in the top line represent FFPE tissue sections after various AR treatments: Low pH, AR solution at low pH value; CAPC, citraconic anhydride solution with boiling; CA98C, citraconic anhydride solution with heating at 98°C; citrate, conventional boiling heating with citrate acid buffer at pH 6.0. Original magnification × 200. Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309. See color insert.

positive, and negative pRB-IHC, respectively. Identical percentages of pRB-positive nuclei were found in all cases, using either of the two protocols for citraconic anhydride or the low pH solution for AR. Inconsistent and significantly weaker nuclear pRB staining results were found when using citrate buffer of pH 6.0 for AR (Table 1.4; Fig. 1.2).

TABLE 1.4 Comparison of pRB-IHC in 27 Cases of FFPE Tissues of Bladder Cancer Using Four Protocols of AR

Cases	AR Protocols	IHC Results			Cases	AR Protocols	IHC Results		
		Intensity	%	Conclusion			Intensity	%	Conclusion
1	CA 98°C	+++	>10	+	12	CA 98°C	++	>10	+
	CA PC	+++	>10	+		CA PC	+++	>10	+
	Low pH	++	>10	+		Low pH	++	>10	+
	Citrate	+	<10	–		Citrate	+	>10	+
2	CA 98°C	+++	>50	++	13	CA 98°C	–	<10	–
	CA PC	+++	>50	++		CA PC	–	<10	–
	Low pH	++	>50	++		Low pH	–	<10	–
	Citrate	+	>10	+		Citrate	–	<10	–
3	CA 98°C	–	<10	–	14	CA 98°C	+	>10	+
	CA PC	–	<10	–		CA PC	++	>10	+
	Low pH	–	<10	–		Low pH	+	>10	+
	Citrate	–	<10	–		Citrate	–	<10	–
4	CA 98°C	–	<10	–	15	CA 98°C	–	<10	–
	CA PC	–	<10	–		CA PC	–	<10	–
	Low pH	–	<10	–		Low pH	–	<10	–
	Citrate	–	<10	–		Citrate	–	<10	–
5	CA 98°C	–	<10	–	16	CA 98°C	–	<10	–
	CA PC	–	<10	–		CA PC	–	<10	–
	Low pH	–	<10	–		Low pH	–	<10	–
	Citrate	–	<10	–		Citrate	–	<10	–
6	CA 98°C	–	<10	–	17	CA 98°C	+++	>50	++
	CA PC	–	<10	–		CA PC	+++	>50	++
	Low pH	–	<10	–		Low pH	+++	>50	++
	Citrate	–	<10	–		Citrate	++	>50	++
7	CA 98°C	++	>10	+	18	CA 98°C	–	<10	–
	CA PC	+++	>10	+		CA PC	–	<10	–
	Low pH	++	>10	+		Low pH	–	<10	–
	Citrate	+	>10	+		Citrate	–	<10	–
8	CA 98°C	–	<10	–	19	CA 98°C	++	>50	++
	CA PC	–	<10	–		CA PC	+++	>50	++
	Low pH	–	<10	–		Low pH	+++	>50	++
	Citrate	–	<10	–		Citrate	++	>10	+
9	CA 98°C	–	<10	–	20	CA 98°C	++	>10	+
	CA PC	–	<10	–		CA PC	+++	>10	+
	Low pH	–	<10	–		Low pH	++	>10	+
	Citrate	–	<10	–		Citrate	+	>10	+
10	CA 98°C	++	>50	++	21	CA 98°C	++	>50	++
	CA PC	+++	>50	++		CA PC	+++	>50	++
	Low pH	+	>50	++		Low pH	++	>50	++
	Citrate	+	>10	+		Citrate	+	>10	+
11	CA 98°C	++	>10	+	22	CA 98°C	–	<10	–
	CA PC	++	>10	+		CA PC	–	<10	–
	Low pH	+	>10	+		Low pH	–	<10	–
	Citrate	±	<10	–		Citrate	–	<10	–

TABLE 1.4 ***Continued***

Cases	AR Protocols	IHC Results			Cases	AR Protocols	IHC Results		
		Intensity	%	Conclusion			Intensity	%	Conclusion
23	CA 98°C	++	>10	+	26	CA 98°C	–	<10	–
	CA PC	++	>10	+		CA PC	–	<10	–
	Low pH	+	>10	+		Low pH	–	<10	–
	Citrate	+	<10	–		Citrate	–	<10	–
24	CA 98°C	–	<10	–	27	CA 98°C	++	>10	+
	CA PC	–	<10	–		CA PC	+++	>10	+
	Low pH	–	<10	–		Low pH	++	>10	+
	Citrate	–	<10	–		Citrate	+	>10	+
25	CA 98°C	++	>50	++					
	CA PC	+++	>50	++					
	Low pH	++	>50	++					
	Citrate	+	>10	+					

Notes: CA98°C, heating tissue sections in 0.05% citraconic anhydride at 98°C for 45 min; CAPC, heating tissue sections in 0.05% citraconic anhydride in a plastic pressure cooker using microwave oven for 30 min; Low pH, heating tissue sections in acetic buffer of pH 1–2 for shorter time as described in the text; Citrate, conventional citrate acid buffer 0.01 M at pH 6.0 with same heating condition of a plastic pressure cooker described above.

Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309.

Third Step: The Western blotting technique, applied to cell extracts, was used to confirm the pRB immunostaining results in two bladder cancer cell lines of T24 and J82, giving quantitative results for pRB in the two cell lines, comparable with that demonstrated by IHC (Fig. 1.3).

Although the novel AR protocol using citraconic anhydride improved the intensity of IHC on FFPE tissue sections for more than half of the antibodies tested, compared to that achieved by other conventional AR protocols, not all antibodies benefitted, which would argue that the citraconic anhydride method does not serve as a truly universal AR protocol. Indeed, many investigators (Table 1.2) have concluded that different antigens may require different "specific" AR protocols. In this respect, the "test battery" is a convenient and cost-effective method for assessing the appropriate AR protocol.[2,8] Nevertheless, the present data certainly support inclusion of the citraconic anhydride AR method in such a "test battery." With respect to the two heating temperatures for citraconic anhydride, the ultimate choice of method for any laboratory may depend on the equipment available.

In a study involving decalcified FFPE rat joint tissue sections and a variety of AR methods, Wilson et al.[32] reported successful application of 0.2 M boric acid at pH 7.0 as the AR solution combining a low-temperature incubation (60°C for 17 h). The principal advantage of this AR protocol was that it minimized lifting or loss of decalcified hard tissue sections from charged slides. Their basic approach for establishing an optimal AR protocol was a "test battery" as described above. In a separate series of studies, based upon prior

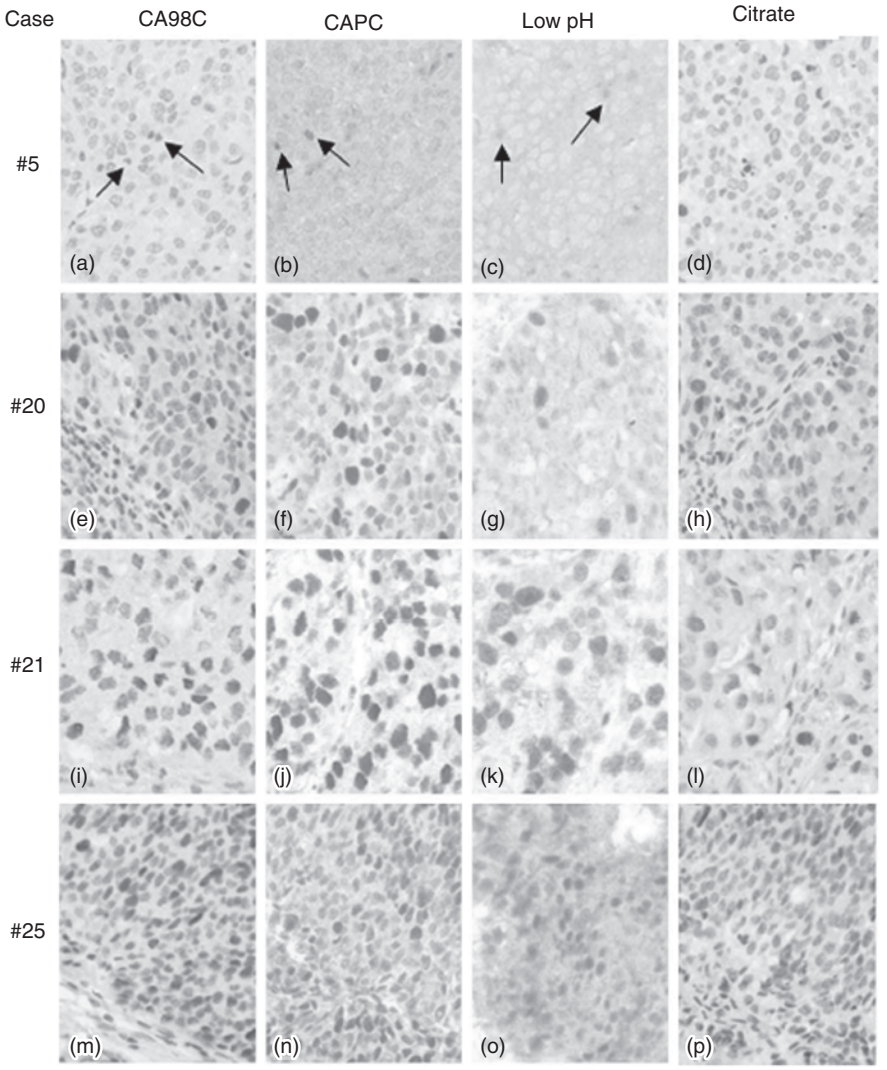

Figure 1.2 Examples of immunostaining intensity from comparison of pRB-IHC in 27 cases of FFPE tissues of bladder cancer (Table 1.4). (A–D) Negative (<10%) showing a few weak positive nuclei (arrows); (E–H) moderate positive (>10%); (I–P) strong positive (>50%). Arrows indicate positive nuclear staining for some lymphocytes or other stromal cells as an internal control. Note the lack of nuclear hematoxylin counterstaining due to low pH AR treatment. The order of cases are indicated in Table 1.4. Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309. See color insert.

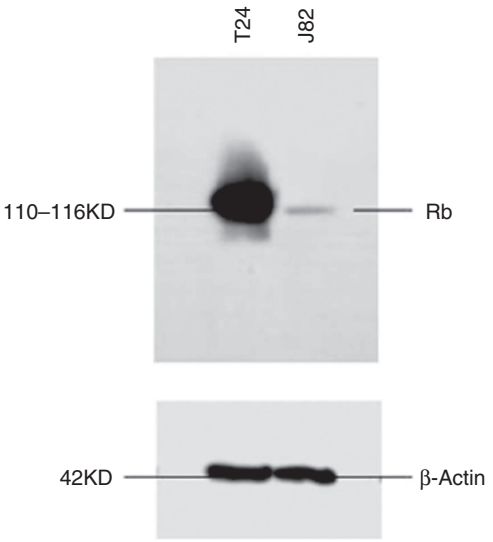

Figure 1.3 Western blotting of pRB protein extracted from two fresh cell lines, T24 and J82. The pRB proteins in fresh T24 cell line showed a stronger band than that obtained from J82 cell line. The Western blotting results correlated well with IHC staining intensity (Table 1.3 and Fig. 1.1). Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309.

literature,[33,34] and with the goal of reducing tissue damage due to boiling during AR, Frost et al.[35] compared a microwave boiling AR protocol and a combination AR protocol that included predigestion in 0.1% trypsin in phosphate-buffered saline (PBS) for 15 min, followed by low-temperature heating in a water bath at 80°C for 2 h. Although tissue damage was reduced by using the low-temperature AR protocol, not all antigens could be recovered equally by this method. They concluded that prior to setting up a new IHC stain, it is critical to assess AR protocols, and primary antibody concentrations as well as detection systems, their standard end point was that method giving the strongest IHC staining signal (maximal retrieval level). In addition, Frost and colleagues also emphasized that the IHC results should be correlated with clinical behavior of diseases in order to provide data that are directly useful for treatment. With a similar principle in mind, Umemura et al.[36] undertook a comparative study of IHC evaluation of hormone receptor status for 861 breast cancer samples with data from IHC and biochemical methods. They demonstrated that optimizing the AR treatment, primary antibodies, and detection systems significantly affects technical validation of IHC data for hormone receptors. They emphasized that the cutoff point should be set higher to reflect the increasing IHC "scores" achieved by more sensitive IHC method, based on the correlation of biochemistry and IHC, as well as clinical follow-up data.

1.2 ANTIBODY AND DETECTION SYSTEM-DEPENDENT TEST BATTERY

Numerous recent articles have emphasized that the application of test battery for establishing an optimal AR protocol is also dependent on the primary antibody and the subsequent detection system. In other words, if an optimal AR protocol is good for antibody clone "1" to protein "A" employing detection system "B," it is not necessarily good for antibody clone 2 to protein A, using the same or different detection systems, but a different AR protocol might give acceptable results. In this respect AR, while in some respects "leveling the playing field" so that many antigens may be detected, in some instances does add yet another variable to achieving consistency among different laboratories. For example, Pan et al.[27] found variable cytoplasmic IHC staining results of TTF-1 for hepatocellular carcinoma, which depended on different sources of the primary antibody and different AR methods. However, they only tested two conditions of AR. Similarly, Slater and Murphy[25] showed great variation in the effectiveness of different AR protocols for IHC staining of an anti-mouse IL-6 antibody (purchased from R&D System, MN, USA) using three AR solutions (pH values of 10.0, 7.0, and 2.0) and four heating conditions (100°C for 10 min, 90°C for 30 min, 80°C for 50 min, 70°C for 1 h). They finally found that there was no staining for IL-6 when using AR solution at pH 10.0 or 7.0 but obtained positive IHC staining at pH 2.0 heated at 80°C for 50 min. Higher temperature heating of 100°C resulted in damage of tissue sections, while lower temperature of 70°C resulted in weak IHC staining.

Again using the test battery principle, Kim et al.[37] compared IHC staining results of two monoclonal antibodies to CD4 (clone: YG23 and 1F6) and three monoclonal antibodies to CD8 (clone: YG20, DN17, and 1A5) on archival FFPE tissue sections using eight different AR solutions at pH values ranging from 2 to 10, combining two heating conditions (heating in a microwave oven vs. heating in a pressure cooker). They found that among five monoclonal antibodies tested, only 1F6 (CD4), and 1A5 (CD8) worked on FFPE tissue sections, and that an AR solution of borate at pH 8.0, containing 1 mM EDTA, and 1 mM NaCl yielded the best IHC staining results for CD4 and CD8. Note, however, that according to their data, it is clear that the use of Tris buffer at higher pH (9–10) also provides satisfactory IHC staining intensity for these two antibodies, a finding having extensive support in the published literature.[14,16,20,21,38–43] Kim et al.[19] also studied seven AR solutions at pH ranging from 2 to 10 for 29 commonly used antibodies and concluded that the optimal AR protocol depends on the particular antibody tested; therefore, the best AR solution should be sought for each antibody, and there is no "universal" approach, nor does AR add reproducibility among laboratories in this context.

Vassallo et al.[44] compared two routinely used antibodies of estrogen receptor (ER), 1D5 (Dakopatts [Carpinteria, CA], code E7101) and 6F11 (Newmarker [Fremont, CA], code MS391-S1) by using two AR protocols,

citrate buffer at pH 6.0 and Tris-EDTA at pH 8.9. For IHC staining, they adopted three different detection systems, EnVision, EnVision Plus, and labeled streptavidin-biotin (LSAB) peroxidase complex (all three systems purchased from Dakopatts). In their study, antibody 6F11, using the citrate AR protocol with EnVision, yielded a poorer IHC signal than that obtained by using Tris–EDTA solution for AR treatment. Kan et al.[45] did a similar comparative study to evaluate the efficacy of different AR protocols, using sodium citrate, citric acid, Tris–HCl, and EDTA buffers of pH 4, 6, and 8, with four different clones of monoclonal antibodies for microtubule-associated protein (MAP)-2-IHC. Staining on FFPE guinea pig brain tissue sections, they found that satisfactory IHC staining was obtained only when MAP-2 antibody clone AP18 was used with the use of AR heating in citric acid buffer of pH 6.0. Gutierrez et al.[46] tested the immunoreactivity of 25 monoclonal antibodies to different leucocyte antigens on FFPE tissue sections, with differing fixation conditions. Employing the test battery approach and the biotin-tyramide amplification system, they concluded that all 25 antibodies tested were readily detectable using an appropriate combination of antibody, AR method, and signal amplification system. Again, no method was optimal for all.

1.3 APPLICATION OF TMA TECHNIQUE FOR TEST BATTERY

Multi-tissue technique has been used for many years in IHC staining to screening numerous samples on one single slide.[47–49] Based on these early observations, TMAs were introduced in IHC for rapid study and to economize in the use of expensive reagents.[50] The TMA technique has the advantage of collecting hundreds of tissue samples on one single slide and provides the additional advantage of increasing the uniformity of staining across the TMA tissues, by reducing diversity of staining signals that result from separate staining of hundreds slides, perhaps on different days, by different technologists. Recent cooperative studies among multiple research centers, such as the BrainNet Europe Consortium, demonstrated the possibility of using the TMA technique in standardization of AR-IHC to achieve reliable results between different laboratories.[51] A multi-tissue "spring-roll" section provided a foundation for standardization of AR-IHC based on giving improved reproducibility and performance of AR-IHC staining results.[52] Camp et al.[53] validated the availability of TMA using three common antigens (ER, progesterone receptor [PR], and HER2) in FFPE tissue sections of invasive breast carcinoma and demonstrated that many proteins retained antigenicity for longer than 60 years using optimal AR pretreatment. Based on numerous studies, a combination of tissue array with AR technique provides an approach to optimize the use of archival FFPE tissue sections with a variety of fields.[54] The advantages are further enhanced by the application of recently developed image analysis software (AQUA) that is designed for quantitative IHC in TMA using an automatic scan.[55]

1.4 SCIENTIFIC ACCURACY OF IHC RELYING ON OPTIMAL AR PROTOCOL

As described above, an optimal AR protocol established by test battery approach produces the best IHC result, defined as the maximal retrieval level (see Chapter 5). It is worthy to note, although not surprising, that not only is "intensity" of staining affected by the choice of the AR method, but also in some cases the distribution and pattern of staining. For example, Mighell et al.[56] demonstrated that fibronectin protein expression pattern, using a polyclonal antibody, was dependent on methods of AR. They used archival FFPE specimens of oral pyogenic granuloma and fibroepithelial polyp, and compared four AR protocols: combinations of enzyme digestion, microwave boiling in citrate buffer, or Tris–HCl buffer at pH 6 or 7.8, and autoclave. They found that after enzyme digestion, there was intense IHC staining in vascular endothelial cells but no staining or minimal staining in connective tissue; in contrast, microwave AR yielded IHC positive staining in connective tissue but no specific vascular staining, while autoclave AR showed positive staining in connective tissue and epithelial nuclei. Comparing these findings with the patterns obtained on frozen tissue sections, there was positive labeling in both vascular endothelial cells and connective tissue. They postulated that different protocols might expose different epitopes. The findings again emphasize the need for optimizing AR for IHC staining in FFPE tissue, while highlighting the concern that AR, when applied without rigorous validation, in fact increases variability observed in IHC staining. Potential causes of these diverse IHC patterns were discussed, including such possibilities as cross-reactivity of the different antibody species within the polyclonal antibody. It is critical to emphasize the fact that variable protein expression patterns may result from different AR protocols, and caution must be taken to avoid misinterpretation. Subsequent published studies obtained somewhat contrasting results.[57,58] Yamashita and Okada[58] studied the mechanism of heat-induced AR employing SDS-PAGE, Western blotting, and IHC. They adopted the same rabbit polyclonal antibody to fibronectin (F-3648, Sigma [St. Louis, MO]) as used by Mighell et al.[56] and found that heating FFPE tissue sections in pH 9.0 buffer solution yielded strong positive fibronectin staining along the basal lamina in the hepatic sinusoid of mouse liver tissue, but no staining when using pH 6.0 buffer. Moreover, they found that boiling FFPE tissue sections in pH 9.0 buffer, followed by heating in pH 6.0 buffer also gave absent or minimal staining. However, boiling the same FFPE slide in pH 9.0 buffer could achieve strong positive staining of fibronectin, suggesting that the pH of AR solution may be an essential factor for proper refolding of epitopes to react with antibodies (see Part IV for details on the study of mechanism of AR).

The generation of artifacts has also been an intermittent concern. Hayashi et al.[59] reported a heat-induced artifact for conversion of Amadori products of the Maillard reaction to N^{ε}-(carboxymethyl) lysine that had the potential to affect IHC staining. However, among thousands of articles pertaining to

numerous antigen/antibody combinations based on AR-IHC in FFPE tissue sections, "false-positive staining" has not been convincingly demonstrated. Nevertheless, caution must be exercised when evaluating a new antibody using AR-IHC staining procedure for FFPE tissue sections. The following issues should be kept in mind to minimize unexpected or spurious staining results: (1) understanding the specificity of the antigen/antibody under test and the distribution in cells/tissues based on information provided by biochemical research; (2) examination of previous IHC staining reports in fresh cell/tissue samples pertaining to this antibody; (3) staining of negative control FFPE tissue section under identical AR treatment but omitting the primary antibody; (4) critical morphological analysis to confirm that observed patterns of distribution are consistent with other known information relating to pathology, molecular biology, and clinical outcome; and (5) in suspicious cases, further confirmation should be sought by using other methods such as Western blotting to confirm the IHC result as emphasized by Wick and Mills.[60]

1.5 ACCURACY OF AR-IHC AS DEMONSTRATED BY IEM AND OTHERS

In recent years, with more accurate quantitative methods, numerous immunoelectron microscopic (IEM) studies have validated the application of AR in archival Epon or other plastic material embedded tissues fixed in aldehyde, plus other fixatives such as osmium tetroxide.[16,57,61–63] Ramandeep et al.[62] designed an interesting study using *Escherichia coli* DH5α cells as a test model, based on quantitative measurements of immunogold labeling IEM, compared to enzyme-linked immunosorbent assay (ELISA) data, to optimize various tissue processing and IEM procedures including AR. They demonstrated that AR can achieve approximately 90–100% retrieval efficiency for osmium-postfixed material, a very interesting finding because cell/tissue samples postfixed with osmium provide the best preservation of ultrastructural morphology for IEM study. Hann et al.[57] carried out a quantitative IEM study based on carefully counting gold labeling particles of collagen IV and fibronectin in the basement membrane underlying the cells of Schlemm's canal from archival aldehyde-fixed LRWhite-embedded eye tissue and found that duration of storage time for archival tissues did not affect AR results. AR did not change the components of the extracellular matrix labeled, and no artifacts were found after AR. They concluded that heat-induced AR can be used on selected extracellular matrix antigens to achieve positive label that would otherwise be lost due to fixation and storage. The test battery approach has also been evaluated by quantitative IEM using gold labeling techniques.[16,17,61] Based on comparison of two polyclonal anti-nestin antibodies, Almqvist et al.[64] demonstrated precise localization of nestin in pediatric brain tumors, previously a controversial issue in the IHC literature. To confirm the reproducibility of counting neurons and glia in human brain tissue sections

by IHC staining, Lyck et al.[26] compared 29 different antibodies with various AR protocols using four buffers (Table 1.2). They reported that it is possible to use IHC staining for reproducible cell counting in brain tissue sections, based on optimal AR protocols, with well-preserved sample materials.

1.6 SUMMARY

- Standardization of AR technique should be based on the test battery principle. Achieving the "maximal retrieval level" of IHC staining intensity is a guideline for standardization.
- Three pH values (acidic, neutral, and basic AR solution), and three heating conditions (under boiling, boiling, and pressure heating) are recommended for the basic "test battery." However, alternative procedures may be applied according to laboratory facilities and routine protocols as described above. Currently, citrate buffer pH 6.0, Tris–EDTA buffer pH 8–9, and certain AR solutions at lower pH, such as boric acid pH 2–3, or acidic acid buffer pH 2, as well as 0.05% citraconic anhydride pH 7.4, may be used to evaluate the optimal AR protocol.
- TMAs are valuable in rapid and cost-effective evaluation of new antibodies, in determining optimal AR methods.

REFERENCES

1. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
2. O'Leary TJ. Standardization in immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* 2001; 9: 3–8.
3. Taylor CR, Levenson RM. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II. *Histopathology* 2006; 49: 411–424.
4. Taylor CR. Standardization in immunohistochemistry: the role of antigen retrieval in molecular morphology. *Biotech. Histochem.* 2006; 81: 3–12.
5. Vani K, Sompuram SR, Fitzgibbons P, et al. National HER2 proficiency test results using standardized quantitative controls. *Arch. Pathol. Lab. Med.* 2008; 132: 211–216.
6. Yaziji H, Taylor CR. Begin at the beginning, with the tissue! The key message underlying the ASCO/CAP task-force guideline recommendations for HER2 testing. *Appl. Immunohistochem. Mol. Morphol.* 2007; 15: 239–241.
7. Wolff AC, Hammond MEH, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. *Arch. Pathol. Lab. Med.* 2007; 131: 18–43.

8. Shi SR, Cote RJ, Yang C, et al. Development of an optimal protocol for antigen retrieval: a "test battery" approach exemplified with reference to the staining of retinoblastoma protein (pRB) in formalin-fixed paraffin sections. *J. Pathol.* 1996; 179: 347–352.
9. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. *J. Histochem. Cytochem.* 1997; 45: 327–343.
10. Shi S-R, Cote RJ, Chaiwun B, et al. Standardization of immunohistochemistry based on antigen retrieval technique for routine formalin-fixed tissue sections. *Appl. Immunohistochem.* 1998; 6: 89–96.
11. Shi S-R, Cote RJ, Taylor CR. Standardization and further development of antigen retrieval immunohistochemistry: strategies and future goals. *J. Histotechnol.* 1999; 22: 177–192.
12. Shi S-R, Gu J, Cote RJ, et al. Standardization of routine immunohistochemistry: where to begin? In *Antigen Retrieval Technique: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 255–272. Natick, MA: Eaton, 2000.
13. Ferrier CM, van Geloof WL, de Witte HH, et al. Epitopes of components of the plasminogen activation system are re-exposed in formalin-fixed paraffin sections by different retrieval techniques. *J. Histochem. Cytochem.* 1998; 46: 469–476.
14. Rocken C, Roessner A. An evaluation of antigen retrieval procedures for immunoelectron microscopic classification of amyloid deposits. *J. Histochem. Cytochem.* 1999; 47: 1385–1394.
15. Shi S-R, Cote RJ, Liu C, et al. A modified reduced temperature antigen retrieval protocol effective for use with a polyclonal antibody to cyclooxygenase-2 (PG 27). *Appl. Immunohistochem. Mol. Morphol.* 2002; 10: 368–373.
16. Yano S, Kashima K, Daa T, et al. An antigen retrieval method using an alkaline solution allows immunoelectron microscopic identification of secretory granules in conventional epoxy-embedded tissue sections. *J. Histochem. Cytochem.* 2003; 51: 199–204.
17. Saito N, Konishi K, Takeda H, et al. Antigen retrieval trial for post-embedding immunoelectron microscopy by heating with several unmasking solutions. *J. Histochem. Cytochem.* 2003; 51: 989–994.
18. Naito I, Ninomiya Y, Nomura S. Immunohistochemical diagnosis of Alport's syndrome in paraffin-embedded renal sections: antigen retrieval with autoclave heating. *Med. Electron Microsc.* 2003; 36: 1–7.
19. Kim SH. Evaluation of antigen retrieval buffer systems. *J. Mol. Histol.* 2004; 35: 409–416.
20. Choi JK, Mauger J, McGowan KL. Immunohistochemical detection of *Aspergillus* species in pediatric tissue samples. *Am. J. Clin. Pathol.* 2004; 121: 18–25.
21. Gill SK, Ishak M, Rylett RJ. Exposure of nuclear antigens in formalin-fixed, paraffin-embedded necropsy human spinal cord tissue: detection of NeuN. *J. Neurosci. Meth.* 2005; 148: 26–35.
22. Du J, Shi XY, Zheng J, et al. Antigen retrieval immunohistochemistry under the influence of pH value and time. *Beijing da Xue Xue Bao. Yi Xue Ban/J. Peking Univ. Health Sci.* 2005; 37: 195–197.
23. Luo X-L, Cai X-L, Liu Y-H, et al. Influence of different antigen retrieval on the immunohistochemistry. *Chinese J. Pathol.* 2005; 34: 52–54.

24. Ge S, Crooks GM, McNamara G, et al. Fluorescent immunohistochemistry and in situ hybridization analysis of mouse pancreas using low-power antigen-retrieval technique. *J. Histochem. Cytochem.* 2006; 54: 843–847.
25. Slater MD, Murphy CR. Co-expression of interleukin-6 and human growth hormone in apparently normal prostate biopsies that ultimately progress to prostate cancer using low pH, high temperature antigen retrieval. *J. Mol. Histol.* 2006; 37: 37–41.
26. Lyck L, Dalmau I, Chemnitz J, et al. Immunohistochemical markers for quantitative studies of neurons and glia in human neocortex. *J. Histochem. Cytochem.* 2008; 56: 201–221.
27. Pan CC, Chen PC, Tsay SH, et al. Cytoplasmic immunoreactivity for thyroid transcription factor-1 in hepatocellular carcinoma: a comparative immunohistochemical analysis of four commercial antibodies using a tissue array technique. *Am. J. Clin. Pathol.* 2004; 121: 343–349.
28. Shi S-R, Cote RJ, Shi Y, et al. Antigen retrieval technique. In *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 311–333. Natick, MA: Eaton, 2000.
29. Hsi ED. A practical approach for evaluating new antibodies in the clinical immunohistochemistry laboratory. *Arch. Pathol. Lab. Med.* 2001; 125: 289–294.
30. Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. *J. Histochem. Cytochem.* 2005; 53: 3–11.
31. Shi S-R, Liu C, Young L, et al. Development of an optimal antigen retrieval protocol for immunohistochemistry of retinoblastoma protein (pRB) in formalin fixed, paraffin sections based on comparison of different methods. *Biotech. Histochem.* 2007; 82: 301–309.
32. Wilson E, Jackson S, Cruwys S, et al. An evaluation of the immunohistochemistry benefits of boric acid antigen retrieval on rat decalcified joint tissues. *J. Immunol. Methods* 2007; 322: 137–142.
33. Elias JM, Margiotta M. Low temperature antigen restoration of steroid hormone receptor proteins in routine paraffin sections. *J. Histotechnol.* 1997; 20: 155–158.
34. Elias JM, Rosenberg B, Margiotta M, et al. Antigen restoration of MIB-1 immunoreactivity in breast cancer: combined use of enzyme predigestion and low temperature for improved measurement of proliferation indexes. *J. Histotechnol.* 1999; 22: 103–106.
35. Frost AR, Sparks D, Grizzle WE. Methods of antigen recovery vary in their usefulness in unmasking specific antigens in immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* 2000; 8: 236–243.
36. Umemura S, Itoh H, Ohta M, et al. Immunohistochemical evaluation of hormone receptor for routine practice of breast cancer: highly sensitive procedures significantly contribute to the correlation with biochemical assays. *Appl. Immunohistochem. Mol. Morphol.* 2003; 11: 62–72.
37. Kim SH, Kook MC, Song HG. Optimal conditions for the retrieval of CD4 and CD8 antigens in formalin-fixed, paraffin-embedded tissues. *J. Mol. Histol.* 2004; 35: 403–408.
38. Pileri SA, Roncador G, Ceccarelli C, et al. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. *J. Pathol.* 1997; 183: 116–123.

39. Evers P, Uylings HB. An optimal antigen retrieval method suitable for different antibodies on human brain tissue stored for several years in formaldehyde fixative. *J. Neurosci. Methods* 1997; 72: 197–207.

40. Shi SR, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J. Histochem. Cytochem.* 1995; 43: 193–201.

41. Shi SR, Cote RJ, Young L, et al. Use of pH 9.5 Tris-HCl buffer containing 5% urea for antigen retrieval immunohistochemistry. *Biotech. Histochem.* 1996; 71: 190–196.

42. Evers P, Uylings HB, Suurmeijer AJ. Antigen retrieval in formaldehyde-fixed human brain tissue. *Methods* 1998; 15: 133–140.

43. Jiao Y, Sun Z, Lee T, et al. A simple and sensitive antigen retrieval method for free-floating and slide-mounted tissue sections. *J. Neurosci. Methods* 1999; 93: 149–162.

44. Vassallo J, Pinto GA, Alvarenga JM, et al. Comparison of immunoexpression of 2 antibodies for estrogen receptors (1D5 and 6F11) in breast carcinomas using different antigen retrieval and detection methods. *Appl. Immunohistochem. Mol. Morphol.* 2004; 12: 177–182.

45. Kan RK, Pleva CM, Hamilton TA, et al. Immunolocalization of MAP-2 in routinely formalin-fixed, paraffin-embedded guinea pig brain sections using microwave irradiation: a comparison of different combinations of antibody clones and antigen retrieval buffer solutions. *Micros. Microanal.* 2005; 11: 175–180.

46. Gutierrez M, Forster FI, McConnell SA, et al. The detection of CD2+, CD4+, CD8+, and WC1+ T lymphocytes, B cells and macrophages in fixed and paraffin embedded bovine tissue using a range of antigen recovery and signal amplification techniques. *Vet. Immunol. Immunopathol.* 1999; 71: 321–334.

47. Battifora H. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. *Lab. Invest.* 1986; 55: 244–248.

48. Wan WH, Fortuna MB, Furmanski P. A rapid and efficient method for testing immunohistochemical reactivity of monoclonal antibodies against multiple tissue samples simultaneously. *J. Immunol. Methods* 1987; 103: 121–129.

49. Lampkin SR, Allred DC. Preparation of paraffin blocks and sections containing multiple tissue samples using a skin biopsy punch. *J. Histotechnol.* 1990; 13: 121–123.

50. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat. Med.* 1998; 4: 844–847.

51. Alafuzoff I, Parkkinen L, Al-Sarraj S, et al. Assessment of [alpha]-synuclein pathology: a study of the BrainNet Europe Consortium. *J. Neuropathol. Exp. Neurol.* 2008; 67: 125–143.

52. Wong SC, Chan JK, Lo ES, et al. The contribution of bifunctional SkipDewax pretreatment solution, rabbit monoclonal antibodies, and polymer detection systems in immunohistochemistry. *Arch. Pathol. Lab. Med.* 2007; 131: 1047–1055.

53. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. *Lab. Invest.* 2000; 80: 1943–1949.

54. Eguíluz C, Viguera E, Millán L, et al. Multitissue array review: a chronological description of tissue array techniques, applications and procedures. *Pathol. Res. Pract.* 2006; 202: 561–568.

55. Cregger M, Berger AJ, Rimm DL. Immunohistochemistry and quantitative analysis of protein expression. *Arch. Pathol. Lab. Med.* 2006; 130: 1026–1030.
56. Mighell AJ, Robinson PA, Hume WJ. Patterns of immunoreactivity to an anti-fibronectin polyclonal antibody in formalin-fixed, paraffin-embedded oral tissues are dependent on methods of antigen retrieval. *J. Histochem. Cytochem.* 1995; 43: 1107–1114.
57. Hann CR, Springett MJ, Johnson DH. Antigen retrieval of basement membrane proteins from archival eye tissues. *J. Histochem. Cytochem.* 2001; 49: 475–482.
58. Yamashita S, Okada Y. Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. *J. Histochem. Cytochem.* 2005; 53: 13–21.
59. Miki Hayashi C, Nagai R, Miyazaki K, et al. Conversion of Amadori products of the Maillard reaction to N(epsilon)-(carboxymethyl)lysine by short-term heating: possible detection of artifacts by immunohistochemistry. *Lab. Invest.* 2002; 82: 795–808.
60. Wick MR, Mills S. Consensual interpretive guidelines for diagnostic immunohistochemistry. *Am. J. Surg. Pathol.* 2001; 25: 1208–1210.
61. Röcken C, Roessner A. An evaluation of antigen retrieval procedures for immunoelectron microscopic classification of amyloid deposits. *J. Histochem. Cytochem.* 1999; 47: 1385–1394.
62. Ramandeep, Dikshit KL, Raje M. Optimization of immunogold labeling TEM: an ELISA-based method for rapid and convenient simulation of processing conditions for quantitative detection of antigen. *J. Histochem. Cytochem.* 2001; 49: 355–367.
63. Saito N, Konishi K, Takeda H, et al. Antigen retrieval trial for post-embedding immunoelectron microscopy by heating with several unmasking solutions. *J. Histochem. Cytochem.* 2003; 51: 989–994.
64. Almqvist PM, Mah R, Lendahl U, et al. Immunohistochemical detection of nestin in pediatric brain tumors. *J. Histochem. Cytochem.* 2002; 50: 147–158.