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Chapter 3

Antigen Retrieval

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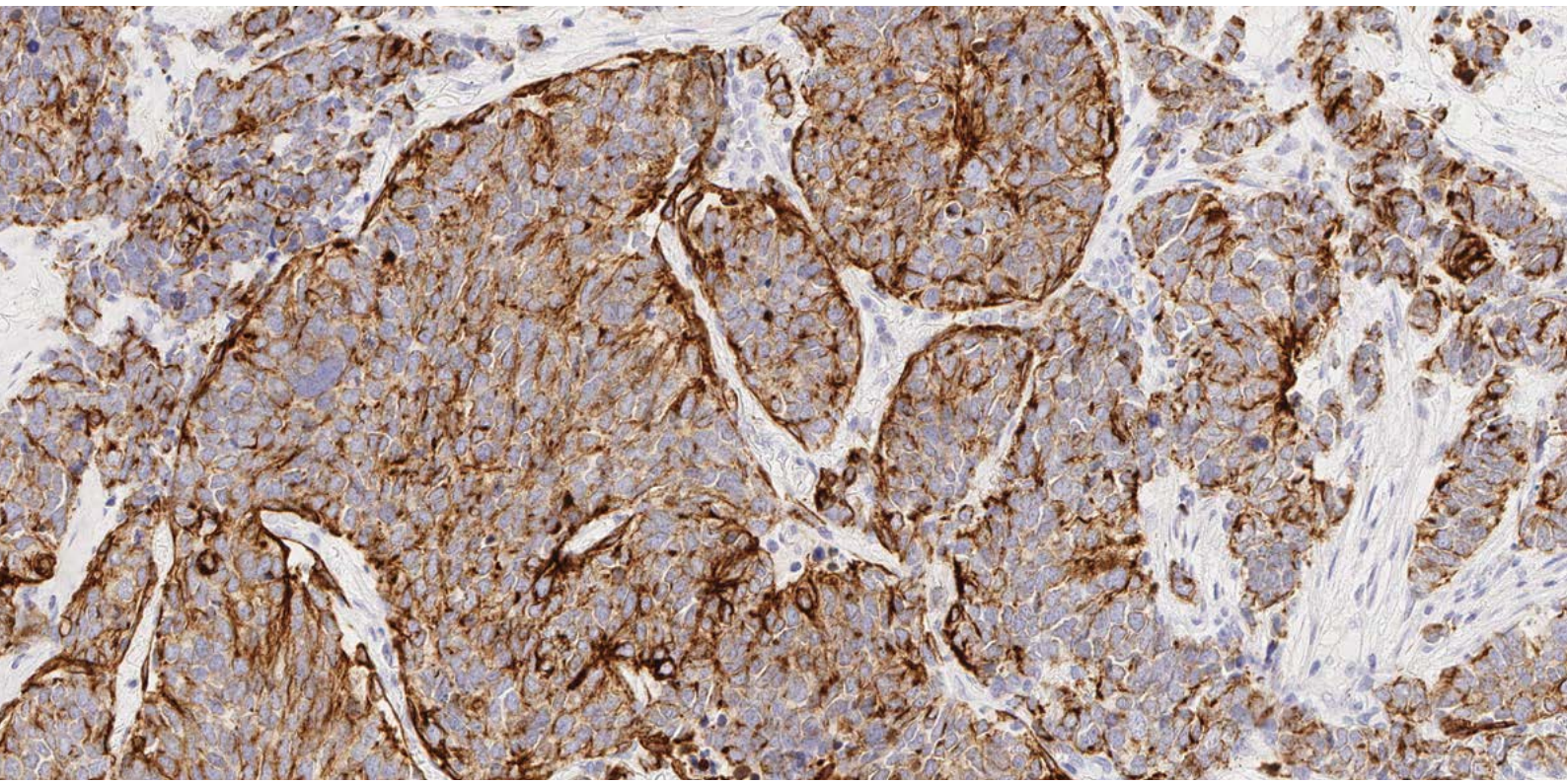

Re•triev•al *(n.)*
The act or process of getting and bringing back something.

Merriam-Webster Online Dictionary

Chapter 3.1 | Introduction

In the majority of cases, tissue specimens for immunohistochemical (IHC) staining are routinely fixed in formalin and subsequently embedded in paraffin. Because of the long history of the use of formalin-fixed, paraffin-embedded (FFPE) tissue sections in histopathology, most of the criteria for pathological diagnosis have been established by the observation of FFPE tissue sections stained by hematoxylin and eosin. Additionally, a great number of FFPE tissue blocks, accompanied by known follow-up data, have been accumulated worldwide, providing an extremely valuable resource for translational clinical research and basic research that cannot easily be reproduced. The major drawback of FFPE tissue is that formalin-induced molecular modification of proteins (antigens) may result in loss of the ability of the antibody to react with the antigen, a loss that can only be corrected by the restoration (retrieval) of the 'formalin-modified' antigen molecular structure. Since the early 1970s, many active pioneers, mostly practicing pathologists who were acutely aware of the need to enhance the capabilities of IHC on FFPE tissue sections while retaining morphologic features, have been searching for an effective retrieval technique (1). Some retrieval methods, such as enzyme digestion, improved IHC staining only for limited antigens. One of the authors (Shi) began a different approach, based upon the practical and theoretical issues to be addressed. A key scientific question was whether fixation in formalin modified the structure of antigens in a reversible or an irreversible manner. To be more specific, was there any prior scientific evidence that the effects of formalin fixation on proteins could be reversed? And if reversed, was the structure of protein restored to a sufficient degree for recovery of antigenicity? With these key questions in mind, Shi spent many days and nights in 1988, prior to online data access, searching the chemical literature the old fashioned way! The answer was finally found in a series of studies of the chemical reactions between protein and formalin, published in the 1940s (2-4). These studies indicated that cross-linkages between formalin and protein could be disrupted by heating above 100 °C, or by strong alkaline treatment. With this knowledge of high temperature heating as a potential retrieval approach, the heat-induced AR technique was developed in 1991 (5).

Subsequently, this AR technique has been applied to in situ hybridization, TUNEL, immunoelectron microscopy, blocking cross-reactions for multiple immunolabeling, aldehyde-fixed frozen tissue sections, mass spectometry on FFPE tissue sections, and the development of a series of novel techniques for successful extraction of nucleic acids and proteins from FFPE tissues (6). Arguably this contribution to protein extraction has proved critical to the development of modern tissue proteomics on FFPE tissues (7, 8).

As a result, FFPE archival tissue collections are now seen as a literal treasure of materials for clinical and translational research, to an extent unimaginable prior to the introduction of heat-induced antigen retrieval two decades ago. The advantages of FFPE tissues in terms of preservation of both morphology and molecules in cell/tissue samples are broadly recognized. For example, there is a growing body of literature demonstrating successful application of FFPE tissue samples for molecular analysis, using AR based methods

Table 3.1 Comparison of frequency concerning application of different terms of heat-induced AR according to OVID Medline data of the 1st week of July & August 2013.

Different terms used	Total articles		Percentage (%)	
	1st week of July	1st week of August	1st week of July	1st week of August
Antigen retrieval	138	140	63.9	63.9
Epitope retrieval	22	22	10.2	10.1
Heat-induced epitope retrieval	15	15	6.9	6.9
Microwave treatment	41	42	19	19.1
Total	216	219	100	100

for extraction of DNA/RNA, and proteins from FFPE tissues. Today, twenty years on, the AR technique is widely, almost universally, used in surgical pathology, including veterinary pathology, in all morphology based sciences, and in pharmacology drug related research, with thousands of original articles published worldwide (6). The enormous impact is reflected in the need to divide all publications with respect to IHC on FFPE tissue into two phases: the pre-AR and post-AR eras, with the dividing line in the early 1990s (9). The term "antigen retrieval" (AR) was originally adopted by Shi and colleagues in 1991. Other terms exist, such as heat-induced epitope retrieval (HIER) or antigen unmasking/demasking, but have no particular merit to cause replacement of the original term (10). Table 3.1 is a comparison of frequency with respect to usage of different terms for this technique. Clearly the original term, antigen retrieval, has greatest acceptance and will be employed in this chapter.

The earlier introduction of enzymatic pre-treatment of tissue sections (11) remains in use for certain selected applications, but these methods are much more difficult to control and have been largely replaced by heat-induced AR.

Chapter 3.2 | Major Factors that Influence the Effect of Antigen Retrieval

Following wide application of the heat-induced AR, numerous modifications of the AR technique and various protocols have been documented in literature. As a result, there is a growing need for standardization of the AR technique itself. The critical importance of standardization of AR-IHC has been emphasized by the American Society of Clinical Oncology and the College of American Pathologists in their Guideline Recommendations for HER2 testing in breast cancer, as well as numerous subsequent documents (12a, 12b, 13). In order to understand the key issues with respect to standardization of AR, it is critical first to study the major factors that influence the effectiveness of AR-IHC. The following conclusions are based on our more than twenty year experience of research, and upon literature review.

- Heating is the most important factor: high temperature heating of formaldehyde-fixed proteins in FFPE tissue sections produces hydrolysis that contributes to break down cross-links (14, 15). In the very first article on AR, Shi and colleagues (5) showed a strong keratin-positive staining result simply by boiling sections in distilled water in a microwave oven. While the composition of the AR solution plays a part, it is the presence of heat and water that is critical: immersing FFPE tissue sections in pure 100% glycerine followed by the IHC staining procedure gives a negative result, adding water to the glycerine and boiling again, gives satisfactory IHC staining (16). That high temperature heating is the most important factor for AR technique has been confirmed by numerous subsequent publications (17, 18). There are several critical technical points with respect to the combination of heating temperature and heating time (heating condition = heating temperature x heating time):
- For many antigens, almost any kind of heating treatment, including microwave oven, water bath, pressure cooker, or autoclave may generate similar results, if adjusted appropriately for time
- There is generally an inverse correlation between heating temperature (T) and heating time (t), as expressed by the formula: AR = T x t (19)
- For most antigens, higher temperature heating, such as boiling FFPE tissue sections for 10-20 minutes, may be an optimal heating condition. However, a few antigens require lower temperature heating conditions, over a longer period of time (20).
- It has been recommended that to preserve tissue morphology, a lower temperature (90 °C) with an elongated time may be preferable (21)
- Within the above generalizations, for some antigens the most extreme conditions of temperature and time (e.g. pressure cooker for hours) gives the greatest staining, but at the cost of morphology. Such methods should be considered as a last resort.

pH Value of the AR Solution

The pH value of the AR solution is another factor that significantly influences the result of AR-IHC. In 1995, we (22) tested the hypothesis that pH of the AR solution may influence the quality of immunostaining of a panel of antibodies, by comparing seven different AR buffer solutions at different

pH values ranging from 1 to 10. The conclusions of this study are relevant when choosing the optimal AR method for any particular antigen/antibody pairing:

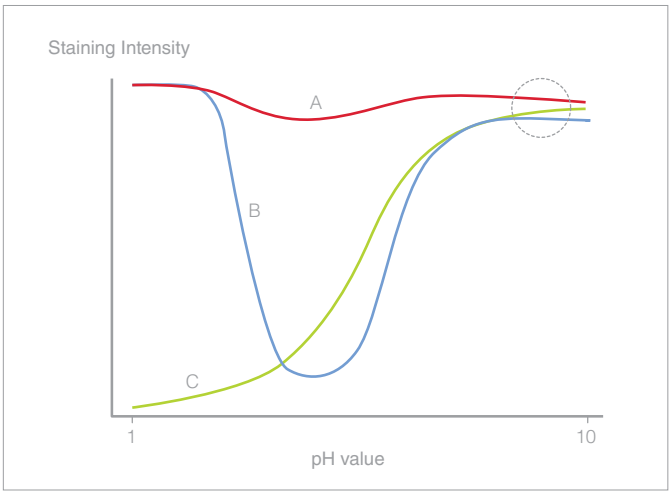

Figure 3.1 Schematic diagram of the three patterns of pH-influenced AR immunostaining. Line A (pattern of Type A) shows a stable pattern of staining with only a slight decrease in staining intensity between pH 3 and pH 6. Line B (pattern of Type B) shows a dramatic decrease in staining intensity between pH 3 and pH 6. Line C (pattern of Type C) exhibits an ascending intensity of AR immunostaining that correlated with increasing pH value of the AR solution. Circle (right) indicates the advantage of using an AR solution of higher pH value. With permission, reproduced from Shi S-R, et al. J Histochem Cytochem 1995;43:193-201.

1. Three types of patterns, reflecting the influence of pH, are indicated in Figure 3.1.
2. **A**, several antigens/clones showed no significant variation utilizing AR solutions with pH values ranging from 1.0 to 10.0 (L26, PCNA, AE1, EMA and NSE); **B**, other antigens/clones (MIB1, ER) showed a dramatic decrease in the intensity of the AR-IHC at middle range pH values (pH 3.0-6.0), but strong AR-IHC results above and below these critical zones; and **C**, still other antigens/clones (MT1, HMB45) showed negative or very weak focally positive immunostaining with a low pH (1.0-2.0), but excellent results in the higher pH range.
3. Among the seven buffer solutions at any given pH value, the intensity of AR-IHC staining was very similar, except that Tris-HCl buffer tended to produce better results at higher pH, compared with other buffers.
4. Optimization of the AR system should include optimization of the pH of the AR solution.
5. A higher pH AR solution, such as Tris-HCl or sodium acetate buffer at pH 8.0-9.0, may be suitable for most antigens (see circle in Figure 3.1).
6. Low pH AR solutions, while useful for nuclear antigens may give a focal weak false positive nuclear staining; the use of negative control slides is important to exclude this possibility.

Numerous investigators have independently reached similar conclusions (23-26).

Chemical Composition of the AR Solution

Other potential factors have been examined for their effect on AR. In considering citrate buffer, it is generally accepted that effectiveness is not dependent so much on the chemical, “citrate”, as upon the high temperature heating. Studies have tested various additives to AR solutions, including metal salts, urea and citraconic anhydride; the last of these showed promise in achieving stronger intensity by testing 62 commonly used antibodies, findings confirmed by others (28, 29). In our comparative study between citrate buffer and citraconic anhydride, using 30 antibodies, more than half (53%) showed a stronger intensity of IHC when using citraconic anhydride for AR, whereas 12 antibodies (43%) gave equivalent results; only one antibody gave a stronger intensity using citric buffer alone for AR (28).

Today many commercial retrieval solutions are available, often as part of an RTU approach to an automated platform (see Chapter 5), and some products contain secret ingredients. Under prescribed conditions many of these reagents give good results, but care should be exercised in applying commercial AR solutions, of unknown composition, to targets other than those described by the vendor, or in protocols other than those recommended; both false positive and false negative results can occur.

With the growing use of automated staining platforms, the choice of ‘autostainer’ to a large degree dictates not only the selection of the primary antibody (see Chapter 4), and its concentration, but also the detection system, and the protocol (see Chapter 5 and Chapter 6), including the method of antigen retrieval. The vendors of automated stainers generally offer recommended AR protocols for (almost) all of the primary antibodies in their portfolio, usually a high pH method (pH 9), a mid/low pH meth-

od (pH 6), and an enzyme-based method for a small number of antibodies. The recommendation usually includes the use of proprietary AR solutions, and defined heating conditions, as part of the protocol. As noted above, departure from these recommendations requires a full revalidation process.

For new antibodies (see Chapter 4), and for antibodies produced by other vendors (other than the manufacturer of the particular automated stainer in use) the laboratory must undertake a study to establish the optimal retrieval method. For this purpose it is recommended that the laboratory use some variation of the Test Battery approach introduced by Shi and colleagues.

Chapter 3.3 | Standardization of AR – The "Test Battery" Approach

In 1996, a "test battery" approach was recommended as a method for quick examination of the two major factors that affect the outcome of AR, namely the heating condition and pH value, in order to reach the strongest signal of AR-IHC (maximal level of AR) (30). This test battery serves as a rapid screening approach to optimize the AR protocol and in so doing achieve some degree of standardization (31). In the initial recommendation the test battery included three levels of heating conditions (below-boiling, boiling and above-boiling), and three pH values (low, moderate, and high), such that a total of nine FFPE tissue sections were used (Table 3.2).

Table 3.2. Test battery suggested for screening an optimal antigen retrieval protocol.

Temperature	**Tris-HCl buffer**		
	pH 1.0-2.0 (Slide #)[a]	pH 7.0-8.0 (Slide #)[a]	pH 10.0-11.0 (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 to 8.0, as the results are similar, and citrate is most widely used.
(b) The temperature of super-high at 120°C may be reached by either auto claving or pressure cooker, 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.
Modified from Shi SR, et al. J. Histochem. Cytochem. 45: 327-343. 1997.

In practice, the method may be further simplified in the following ways;

- Test three pH values by using one temperature (boiling), select the best pH value and then test various temperatures; or,
- Test several commonly used AR solutions (or those recommended for the autostainer in use in the laboratory), such as citrate buffer pH 6.0, Tris-HCl + EDTA of pH 9.0

Although this later method is not a complete test, it is more convenient for most laboratories. If satisfactory results are not obtained other variations may be tested, including citraconic anhydride, or enzyme-based digestion methods. 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.

Specially prepared tissue microarrays (TMAs), incorporating a range of tissues and tissue cores fixed for differing times, are also of value in helping establish the optimal AR method for a particular antibody, by staining of only a few TMA slides. The advantages are further enhanced by application of recently developed image analysis software (AQUA) that is designed for quantitative IHC in TMA using an automatic scan (32).

Table 3.3 Major applications of antigen retrieval technique and principle.

Areas of application of AR	Application of AR technique and/or principle	Reference
Immunoelectron microscopy (IEM)	AR pre-treatment of Epon-embedded ultra-thin sections after etching the grids by solutions[(a)] to achieve satisfactory positive results; or, directly heating the grid and followed by washing procedures including 50 mM NH_4Cl and 1% Tween 20	39, 40
In situ hybridization (ISH)	High temperature heating FFPE tissue sections prior to ISH to achieve satisfactory results	41-43
TUNEL	Optimal heating time, as short as 1 min to improve the signal	44, 45
Multiple IHC staining procedures	Adding a microwave heating AR procedure (10 min) between each run of the multiple IHC staining procedure effectively blocks cross-reactions, by denaturing bound antibody molecules from the previous run	33
Human temporal bone collections	Combining sodium hydroxide-methanol and heating AR treatment provides an effective approach for IHC used in celloidin-embedded temporal bone sections. This method is also used for plastic-embedded tissue sections, including IEM	46, 47
Immunofluorescence	To enhance intensity and reduce autofluorescence	48
Cytopathology	AR pre-treatment of archival PAP smear slides promotes satisfactory IHC staining	49
Flow cytometry (FCM)	Enzyme digestion followed by heating AR treatment was adopted to achieve enhancement of FCM of FFPE tissue	50
Floating vibratome section	Microwave boiling of vibratome sections improves IHC staining results; further extended for use with whole mount tissue specimens	51
En Bloc tissue	AR heating of 4% paraformaldehyde-fixed animal brain or testis tissue blocks enhances immunoreactivity for most antibodies tested	52
Frozen tissue section	Aldehyde-fixed frozen tissue section with use of AR treatment gives both excellent morphology and IHC staining	34, 35
DNA extraction from FFPE tissue sections	Boiling AR pre-treatment prior to DNA extraction gives improved results compared to enzyme treatment	53-56
RNA extraction from FFPE tissue sections	Heating AR treatment prior to RNA extraction gives improved results compared to enzyme treatment	57, 58
Protein extraction from FFPE tissue sections	AR pre-treatment with AR solution including 2% SDS and/or other chemicals improves efficiency of protein extraction from FFPE tissue compared to enzyme digestion. Combining with elevated hydrostatic pressure may increase extraction of up to 80-95% of proteins from FFPE tissue sections	59-62
Imaging mass spectrometry (IMS)	AR pre-treatment gives satisfactory results of IMS . Based on comparison among different AR solutions, Gustafsson et al summarized that citrate acid AR method is an important step in being able to fully analyze the proteome for FFPE tissue	36-38

AR = antigen retrieval; FFPE = formalin-fixed paraffin-embedded; IEM = immunoelectron microscopy; ISH = in situ hybridization; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling; FCM = flow cytometry; IMS = imaging mass spectrometry. (a) 10% fresh saturated solution of sodium ethoxide diluted with anhydrous ethanol for 2 min or with a saturated aqueous solution of sodium metaperiodate for 1 hour. Reprinted with permission from Shi SR, et al. J. Histochem. Cytochem. 59:13-32, 2011.

Chapter 3.4 | Application of AR Techniques – The Basic Principles

In addition to its use in IHC, AR has increasingly been adopted in the following related applications:

- In situ hybridization (ISH) and in situ end-labeling (TUNEL) of apoptotic cells in FFPE tissue sections; as well as in flow cytometry to achieve stronger positive signals while reducing non-specific background noise
- In IHC multi-stains, AR has been used to block the cross-reaction from the previous run (33)
- In addition to FFPE tissue sections, AR has been adopted for aldehyde-fixed fresh tissue sections, plastic-embedded tissue sections, cell smear samples for cytopathology, and floating vibratome sections (33)
- Modified AR methods have been used successfully for extraction of DNA and RNA from FFPE tissue sections for PCR-based methods and sequencing
- Imaging mass spectrometry (IMS) has been applied to proteins extracted from FFPE tissue sections by AR approaches, providing an avenue to fully analyze the proteome of archival FFPE tissue (36-38)

Chapter 3.5 | AR-IHC-based Research and Diagnostics

Over the past two decades AR has found extensive application, not only for IHC, but also for molecular methods applied to FFPE tissues, so called tissue proteomics, as well as standardization and quantification of IHC. For further details the reader is referred to the multi-author text edited by Shi and colleagues (6), which includes discussion of a proposal for quantitative IHC, based upon the use of AR. This hypothesis proposes to minimize the variation in IHC that is observed in clinical FFPE tissue sections, by using optimal antigen retrieval (AR) in a test battery approach. The intent is to use AR to reduce the loss of antigenicity observed for many proteins, following variable fixation, to a level comparable to frozen tissue sections, at which point a standard calibration curve could be developed using internal proteins. This approach is similar to that of enzyme-linked immunosorbent assays (ELISA) where a standard curve is used to convert the immunoreaction signal into a quantitative amount of protein (63).

Chapter 3.6 | Reagents and Protocols

Sections 3.6-3.12 will describe the following retrieval protocols:

- Water Bath Methods
  - Dako PT Link
  - Water Bath (conventional) Heating
- Pressure Cooker Heating
- Autoclave Heating
- Microwave Oven Heating
- Proteolytic Pre-treatment
- Combined Proteolytic Pre-treatment and Antigen Retrieval
- Combined Deparaffinization and Antigen Retrieval

The composition and the pH of retrieval buffers are crucial for optimal retrieval. Although citrate buffers of pH 6 are widely used retrieval solutions, high pH buffers have been shown to be widely applicable for many antibodies, as discussed previously (22, 64). It is the responsibility of the individual laboratory to determine which of the available buffers perform optimally for each antigen/antibody and then to use them consistently. Although 0.01 M citrate buffers of pH 6 have historically been the most widely used retrieval solutions, high pH buffers have started being implemented when showing improved end results for some antigens. The following protocol descriptions should serve as guidelines only. It is the responsibility of the individual laboratory to compare methods and select the optimal protocol for consistent use. It is recommended for the AR methods to control temperature settings and to measure the actual temperature at regular intervals. The following protocols focus mostly on Dako reagents and systems, with detailed input from Dako; other manufacturers supply similar reagents and protocols, which should be followed scrupulously.

Chapter 3.7 | Water Bath Methods

A. Dako PT Link

Dako PT Link instrument simplifies the water bath antigen retrieval process by performing automated retrieval using specified protocols, which incorporate preheat temperature, antigen retrieval temperature, and time as well as cool down settings. Typically, antigen retrieval is performed for 20 minutes at 97 °C.

Figure 3.2 Dako PT Link is a water bath method for antigen retrieval

Materials Required

- Dako PT Link*
- Dako Autostainer Slide Rack
- Retrieval solution
- FLEX IHC Microscope Slides or slides coated with other suitable adhesives
- Personal protective equipment

*Dako Omnis has onboard pre-treatment module. See User Manual for protocol.

Protocol

Wear chemical-protective gloves when handling parts immersed in any reagent used in PT Link.

1. Deparaffinize and rehydrate tissue sections.
2. Prepare a working solution of the selected target retrieval solution according to specifications.
3. Fill tanks with 1.5 L of desired target retrieval solution.
4. Place tank lids on tanks. Close and lock main lid with external latch.
5. See Operator's Manual for instrument set-up details:
   a. Recommended time is 20-40 minutes.
   b. Set antigen retrieval temperature to 97 °C.
   c. Set preheat temperature to 65 °C (allows up to 85 °C).
6. Press [RUN] button for each tank and the CYCLE will show PREHEAT. Allow solution to reach the selected preheat temperature.
7. Open the PT Link and immerse the Autostainer Slide Rack with deparaffinized tissue sections into the preheated target retrieval solution.*
8. Place tank lids on tanks. Close and lock main lid with external latch.
9. Press [RUN] button for each tank to start run. CYCLE will show WARM-UP and the lid lock will engage.
10. PT Link will warm up to preset temperature and then start the countdown clock for target retrieval cycle.
11. When target retrieval cycle is finished, CYCLE will show COOL. The COOL cycle is finished when temperature reaches Preheat SET temperature, even if Preheat is disabled.
12. When COOL cycle is finished, CYCLE will show DONE and lid will unlock automatically.
13. Open the PT Link and remove each slide rack with the slides from the PT Link Tank and immediately immerse slides into the PT Link Rinse Station containing diluted, room temperature Dako Wash Buffer (10x).
14. Leave slides in the diluted, room temperature Dako Wash Buffer for 1-5 minutes.
15. Proceed with IHC staining.

*As an alternative, a 3-in-1 solution can be used for both deparaffinization and target retrieval. See Section 3.13 I Combined Deparaffinization and Antigen Retrieval.

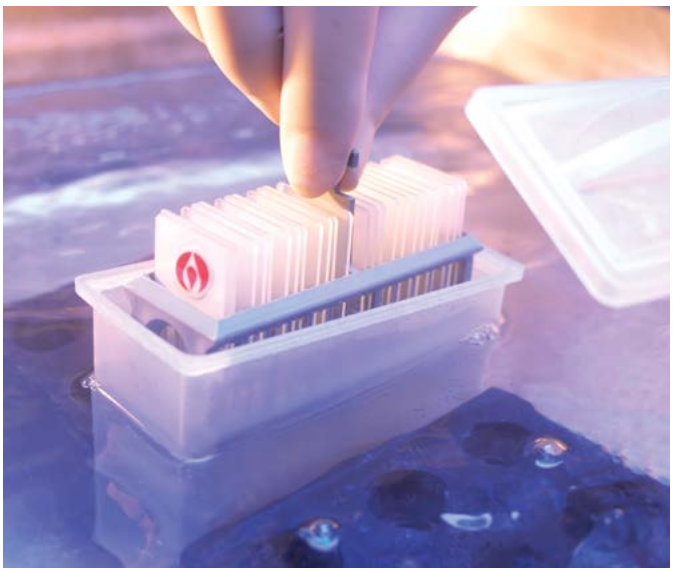

Figure 3.3 Antigen retrieval in conventional water bath.

B. Water Bath (conventional) Heating

One of several advantages of the water bath heating method is the ready availability of water baths in most clinical laboratories. Temperature settings just below the boiling point of water (95-99 °C) are most commonly used.

Materials Required

- Temperature-controlled water bath
- Slide rack
- Incubation container and cover
- Retrieval solution
- Tris-Buffered Saline
- Silanized Slides or slides coated with other suitable adhesives
- Thermometer
- Personal protective equipment

Protocol

It is recommended to wear insulated gloves when handling parts immersed in any reagent used in a water bath.

1. Deparaffinize and rehydrate tissue sections.
2. Fill container with enough retrieval solution to cover slides and equilibrate to 95-99 °C in water bath.
3. Immerse racked slides in preheated retrieval solution, cover container with lid, and incubate for specified time within the 20-40 minutes range after the set temperature has been reached.
4. Remove the container from the water bath and cool the contents with the lid in place for 20 minutes at room temperature.
5. Rinse with Tris-Buffered Saline (TBS) or distilled water at room temperature.
6. When removing the slides from the container it is very important that the slides do not dry out.
7. Transfer slides to TBS.
8. Proceed with IHC staining.

Chapter 3.8 | Pressure Cooker Heating

Pressure cookers set to approximately 103 kPa/15 psi will achieve a temperature of approximately 120 °C at full pressure. Alternatively, setting at 125 °C can be used for antigen retrieval. Stainless steel pressure cookers are recommended as the aluminum models are susceptible to corrosion by some retrieval solutions. As an alternative, individual plastic container(s) can be filled with retrieval solution and placed in reagent grade water in the pressure cooker pan.

Figure 3.4 Pressure cooker for antigen retrieval.

Materials Required

- Stainless steel pressure cooker, preferably electrically programmable
- Metal or plastic slide racks
- Retrieval solution
- Silanized Slides or slides coated with other suitable adhesives
- Tris-Buffered Saline
- Incubation container (optional)
- Personal protective equipment

Protocol

It is recommended to wear a safety face shield and insulated gloves.

1. Deparaffinize and rehydrate tissue sections.
2. Fill the pressure cooker with enough retrieval solution to cover slides. Alternatively, fill individual plastic container(s) with retrieval solution and add at least 500 mL of reagent grade water to pressure cooker chamber.

3. Bring contents to near boiling point, place racked slides into retrieval solution, seal the pressure cooker, and again bring the solution to a boil. For programmable pressure cookers, set target temperature and heating time, place racked slides in retrieval solution, seal the pressure cooker, and begin antigen retrieval procedure from room temperature.
4. Boil for 30 seconds to 5 minutes and allow the pressure cooker to cool for 20 minutes prior to opening. (Note: Vent any residual pressure before opening). Open programmable pressure cooker when antigen retrieval procedure is completed.
5. Transfer slides to room temperature Tris-Buffered Saline. When removing the slides from the container it is very important that the slides do not dry out.
6. Proceed with IHC staining procedure

Chapter 3.9 | Autoclave Heating

When set to 15 psi, an autoclave, like a pressure cooker, will achieve a temperature of about 120 °C at full pressure (65, 66).

Materials Required

- Bench top autoclave
- Plastic or metal slide rack
- Incubation container
- Retrieval solution
- Silanized Slides or slides coated with other suitable adhesives
- Tris-Buffered Saline
- Personal protective equipment

Protocol

It is recommended to wear safety face shield and insulated gloves.

1. Deparaffinize and rehydrate tissue sections.
2. Place slides in plastic or metal slide rack.
3. Fill the incubation container with enough retrieval buffer (typically 250 mL) to cover slides. Insert the slide rack and cover.
4. Place the container in the autoclave and follow Autoclave Manufacturer's Operating Instructions.
5. Set the temperature to 120 °C/15 psi and the time to 10-20 minutes. Start operation.
6. After venting pressure, open the lid and remove the slide container from the autoclave.
7. Rinse slides in Tris-Buffered Saline (TBS) or reagent grade water. When removing the slides from the container it is very important that the slides do not dry out.
8. Transfer slides to TBS.
9. Proceed with IHC staining procedure.

Chapter 3.10 | Microwave Oven Heating

Microwave ovens are very efficient for the heating of aqueous solutions, however, the standardization of procedures is important when used for antigen retrieval (and for the retrieval of DNA for in situ hybridization, i.e. target retrieval). In an effort to maintain consistency of AR protocols and to ensure reproducibility of staining results, the following elements should be standardized:

- Wattage of the microwave oven
- Presence of a turntable
- Volume of retrieval buffers per container
- Number of slides per container
- Number of containers

Materials Required

750-800 W microwave oven with turntable (please note that the effective power may decrease over time)

- Incubation container for microwave oven
- Plastic slide holder for microwave oven
- Retrieval solution
- Silanized Slides or slides coated with other suitable
- adhesives
- Tris-Buffered Saline
- Personal protective equipment

Protocol

Never use the microwave oven with metallic material present. It is recommended to wear insulated gloves when handling parts immersed in any reagent.

1. Deparaffinize and rehydrate sections.
2. Place slides in slide holder. Fill empty positions with blank slides.
3. Fill incubation container with enough retrieval solution to cover slides and insert slide holder.
4. Cover the container to minimize evaporation. Use a lid with minimal opening to avoid build-up of pressure and reduce evaporation.

5. Place container in the middle of the turntable and heat to near boiling point.
6. Incubate for fixed amount of time, typically 10 minutes.
7. Remove the container from the microwave oven, remove the lid, and allow to cool at room temperature for 15-20 minutes.
8. Rinse with distilled water.
9. Place in Tris-Buffered Saline.
10. Proceed with staining protocol.

Chapter 3.11 | Proteolytic Pre-treatment

As with other pre-treatment methods, procedures for proteolytic pre-treatment vary due to laboratory-specific differences in formalin fixation. Proteolytic pre-treatment must be optimized (dilution and time – specific elevated temperature may also be selected) according to the particular fixation process used in each laboratory. Examples of antigens most often treated with proteolytic enzymes include cytokeratins and immunoglobulins.

Materials Required

- Humidity chamber
- Silanized Slides or slides coated with other suitable adhesives
- Proteolytic Enzyme, Ready-to-Use
- Tris-Buffered Saline

Protocol

1. Deparaffinize and rehydrate tissue sections.
2. Place slides horizontally and apply enough enzyme working solution to cover tissue section(s), typically 200-300 µL.
3. Incubate for defined time, typically 5-15 minutes.
4. Stop enzymatic reaction by rinsing with distilled water or Tris-Buffered Saline.
5. It is recommended that enzyme digestion is included in the relevant Autostainer protocols. For the RTU series antibodies, enzyme digestion is included.

For Dako Proteolytic Enzymes, the following guidelines apply:

Proteinase K, Concentrated and Ready-to-Use:

Digestion for 6 minutes at room temperature is generally sufficient, but may be prolonged to 15 minutes.

Pepsin:

Digestion for 10 minutes at room temperature is generally sufficient, but may be prolonged to 15 minutes.

Proteolytic Enzyme, Ready-to-Use:

Digestion for 5-10 minutes at room temperature is sufficient. For details, please refer to the product specification sheets.

Chapter 3.12 | Combined Proteolytic Pre-treatment and Antigen Retrieval

Some antigens are more efficiently retrieved by a combination of heating and enzyme digestion, e.g. some cytokeratins and immunoglobulin light chains. The protocol below describes a method of first treating with Proteinase K and then AR by either water bath or microwave method.

Materials Required

- Silanized Slides or slides coated with other suitable adhesives
- Target Retrieval Solution, pH 6, Dako*
- Tris-Buffered Saline
- Tris-buffered NaCl Solution with Tween 20 (TBST), pH 7.6

*Other target retrieval solutions will work with a similar protocol optimized according to individual laboratory requirements.

Protocol

It is recommended to wear insulated gloves when handling parts immersed in any reagent.

1. Deparaffinize and rehydrate tissue sections.
2. Cover tissue sections with Proteinase K and incubate for 5-10 minutes.
3. Rinse with distilled water and place in Tris-Buffered Saline.
4. Proceed to antigen retrieval using either PT Link, another water bath or microwave method below.

AR – Water Bath

5. Fill container with enough retrieval solution (200 mL) to cover slides and equilibrate to 95-99 °C in water bath. Place the incubation container into the water bath and incubate for 20-40 minutes.
6. Remove the container from the water bath and cool the contents with the lid removed for 20 minutes at room temperature.

7. Rinse with Tris-Buffered Saline (TBS) or distilled water at room temperature.
8. Transfer slides to Tris-Buffered NaCl Solution with Tween 20 (TBST), pH 7.6 Wash Buffer.
9. Proceed with IHC staining.

AR – Microwave

5. Fill incubation container with enough retrieval solution (200 mL) to cover slides and insert slide holder. Insert slides in holder and cover.
6. Place the incubation container into microwave oven and incubate for 2 x 5 minutes.
7. In between steps 4 and 5, fill up the container with enough distilled water (50 mL) to cover slides.
8. After the second treatment, leave the sections in the retrieval solution at room temperature to cool for 15-20 minutes.
9. Rinse with distilled water.
10. Proceed with IHC staining.

Chapter 3.13 | Combined Deparaffinization and Antigen Retrieval

Combining deparaffinization and AR reduces slide handling time significantly and provides added convenience without sacrificing staining quality. Using Dako PT Link instrument simplifies the combined deparaffinization and target retrieval process by performing automated deparaffinization and retrieval in a single step.

Materials Required

- PT Link
- PT Link Rinse Station
- Silanized Slides or slides coated with other suitable adhesives
- Target Retrieval Solution, pH 9, 10x Concentrated, (3-in-1)*
- Dako Wash Buffer (10x)

*When used in PT Link for 3-in-1 specimen preparation procedure, the diluted deparaffinization / target retrieval solution can be used three times within a five day period, if stored at room temperature.

Table 3.4 Dako Products for Antigen Retrieval**

Product Name	Dako Code
Target Retrieval Solutions	
FLEX Target Retrieval Solution, High pH	K8004
FLEX Target Retrieval Solution, Low pH	K8005
Target Retrieval Solution, pH 6.1, 10x Concentrated	S1699
Target Retrieval Solution, pH 6.1, Ready-to-Use	S1700
Target Retrieval Solution, pH 9, 10x Concentrated	S2367
Target Retrieval Solution, pH 9, Ready-to-Use	S2368
Target Retrieval Solution, pH 9, 10x Concentrated, (3-in-1)	S2375
Proteolytic Enzymes	
Proteinase K, Concentrated	S3004
Proteinase K, Ready-to-Use	S3020
Pepsin	S3002
Proteolytic Enzyme, Ready-to-Use	S3007
Buffers	
Dako Wash Buffer (10x)	S3006
Tris-Buffered Saline	S3001
Tris-buffered NaCl Solution with Tween 20 (TBST), pH 7.6, 10x Concentrated	S3306
Instruments and Other Products	
Dako Omnis	GI100
Dako PT Link	PT100/PT101
PT Link Rinse Station	PT109
PT Link Tank	PT102
Dako Autostainer Slide Rack	S3704
FLEX IHC Microscope Slides	K8020
Silanized Slides	S3003

**Note that other manufacturers provide similar products; the user should bear in mind that commercial products generally are designed and tested to be used in the specified format, within a defined protocol, and specified instrumentation. Products are not freely interchangeable across detections systems, and any change from the recommended protocol requires complete revalidation.

Protocol

Wear chemical-protective gloves when handling parts immersed in any reagent used in PT Link. Recommended 3-in-1 specimen preparation procedure using PT Link and above target retrieval solution:

1. Prepare a working solution of the selected target retrieval solution according to the specifications.
2. Fill PT Link Tanks with sufficient quantity (1.5 L) of working solution to cover the tissue sections.
3. Set PT Link to preheat the solution to 65 °C.
4. Immerse the mounted, formalin-fixed, paraffin-embedded tissue sections into the preheated target retrieval solution (working solution) in PT Link Tanks and incubate for 20-40 minutes at 97 °C. The optimal incubation time should be de termined by the user.
5. Leave the sections to cool in PT Link to 65 °C.
6. Remove each Autostainer Slide Rack with the slides from the PT Link Tank and immediately dip slides into a jar/tank (PT Link Rinse Station) containing diluted, room temperature Dako Wash Buffer (10x).
7. Leave slides in the diluted, room temperature Wash Buffer for 1-5 minutes.
8. Place slides on an automated instrument and proceed with staining. The sections should not dry out during the treatment or during the immunohistochemical staining procedure.
9. After staining, it is recommended to perform dehydration, clearing and permanent mounting.

Chapter 3.14 | Conclusion

As discussed above, an effective AR protocol is based on the major factors that influence the effect of AR-IHC. Thus, for new antibodies, a test battery approach is recommended for establishing the optimal AR protocol for each antigen/antibody pair in FFPE tissue sections. Although citrate buffer of pH 6 is a widely used retrieval solution, high pH buffers have been shown to be widely applicable for many antibodies. It is the responsibility of the individual laboratory to determine which of the listed AR solutions perform optimally for each antigen/antibody pair. In an automated system a new antibody can be ‘plugged’ into an existing automated protocol, and run with whatever two or three choices of antigen retrieval are programmed for the instrument, with the appropriate AR recommended reagents. If satisfactory results are not obtained, it is advised then to revert to a test battery approach.

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