

# Retrieval of antigenic determinants

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Many antigens will become detectable in routinely processed tissues or at least its detection will be improved when sections are pretreated by one of the so-called “antigen retrieval methods”. A number of strategies of antigen retrieval have been published to facilitate immunohistology in histopathology. The first approach was the introduction of proteolytic digestion of tissue sections (HUANG SN, 1975; HUANG SN et al., 1976; CURRAN RC and GREGORY J, 1977; CURRAN RC and GREGORY J, 1978; DENK H et al., 1977; PINKUS GS et al., 1985; MEPHAM BL et al., 1979; BATTIFORA H and KOPINSKI M, 1986; ORDONEZ NG et al., 1988). In a similar way, histological lectin staining could be enhanced by pretreatment of tissue sections with proteolytic enzymes (KUHLMANN WD and PESCHKE P, 1984).

Other proposals for unmasking antigens included hydrochloric acid, formic acid, citraconic anhydride, SDS, urea, and microwaves (HAUSEN P and DREYER C, 1982; COSTA PP et al., 1986; KITAMOTO T et al., 1987; GALAND P and DEGRAEF C, 1989; SHI SR et al., 1991; BROWN D et al., 1996; NAMIMATSU S et al., 2005). Principles of protein masking and unmasking are well known (review by MEANS GE and FEENEY RE, 1971). Before the above methods have come under focus, earlier studies have shown that chemical modification of proteins in the course of tissue processing can be a useful measure for protection of antigenic determinants. The latter are then readily exposed for subsequent immunostaining by appropriate treatment of tissue sections. For example, imidoesters (ethyl acetimidate) can be used for reversal chemical modification of proteins after aldehyde fixation. The method will protect free amino groups by their conversion into unreactive amidines. Prior to immunostaining, acetimidyl groups are then removed by treatment of tissue sections with ammonia-acetic acid. In this approach, antigenic sites become readily exposed (KUHLMANN WD and KRISCHAN R, 1981).

The systematic study of SR SHI et al. (1991) on antigen retrieval in formalin fixed and paraffin embedded tissues by microwave heating of tissue sections could prove the great potential of heat-induced epitope retrieval in immunohistology. This was the beginning of wide-spread application of immunohistology in histopathology. It is thought that retrieval techniques eliminate masking proteins and break protein cross-links which have been formed during fixation and thereby uncover hidden antigenic epitopes.

Just as many histopathological laboratories exist as many different experiences have been collected by using antigen retrieval methods. From all the chemical, proteolytic and heat retrieval methods, two major trends for antigen unmasking in formalin fixed and paraffin embedded specimens can be observed: (a) proteolytic-induced epitope retrieval (PIER), and (b) heat-induced epitope retrieval. \*

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\* Reagents for chemical, proteolytic and heat-induced epitope retrievals can be toxic. They must be handled with care

## Antigen unmasking by proteolytic-induced epitope retrieval (PIER)

Proteolytic enzymes such as pronase, proteinase K, trypsin, papain, pepsin etc. can have antigen-unmasking effects on formaldehyde fixed tissue sections. The required enzyme concentrations, digestion times and reaction temperatures will largely depend on both the antigens and the prepared tissues. The standardization of fixation is important for consistent results. Enzyme digestion should be strictly controlled, otherwise no antigen retrieval is achieved or damage of tissue sections and false negative results will occur. Here, we give digestion schedules for the most preferred proteases; be aware that trypsin digestion in particular can cause tissue damage.

### Pronase (Protease Type XIV) method

Reagents	Solutions
Pronase E, Protease type XIV from <i>Streptomyces griseus</i> (Sigma Chemical Co.) Phosphate buffered saline (PBS) PBS supplemented with 0.1 M glycine (PBS/glycine) PBS supplemented with 1% BSA (PBS/BSA)	<ul style="list-style-type: none"> <li>0.05% Pronase solution: 0.005 g Pronase dissolved in 10.0 mL PBS</li> <li>0.1% Pronase solution: 0.01 g Pronase dissolved in 10.0 mL PBS</li> </ul>
<b>PIER procedure</b> Deparaffinize and rehydrate sections (see chapter <i>Immunostaining with paraffin embedded tissue sections</i> ): <ul style="list-style-type: none"> <li>– Pronase solution (0.05 or 0.1%)                      2-10 min at 37°C (optimal enzyme concentrations and incubation times depend on tissue specimen)</li> <li>– cool down to room temperature                      10 min</li> <li>– distilled water    several rinses</li> <li>– PBS, PBS/glycine or PBS/BSA                      3 x 2 min (optionally)</li> </ul> Perform immunostaining according to standard protocol	

### Proteinase K method

Reagents	Solutions
Proteinase K from <i>Tritirachium album</i> (Sigma Chemical Co.) Tris (TRIS Base) EDTA (disodium EDTA) HCl (ca 10-30%) Phosphate buffered saline (PBS) PBS supplemented with 1% BSA (PBS/BSA)	<ul style="list-style-type: none"> <li>Tris-EDTA buffer pH 8.0 (0.05 M Tris and 0.001 M EDTA): 6.06 g Tris <i>plus</i> 0.37 g EDTA are dissolved in 1000.0 mL distilled water</li> <li>mix and adjust pH to 8.0 with concentrated HCl</li> <li>0.04% Proteinase K stock solution:</li> </ul>

Distilled water	<p>4.0 mg Proteinase K are dissolved in 10.0 mL Tris-EDTA buffer</p> <ul style="list-style-type: none"> <li>• Proteinase K working solution (0.002%): 1.0 mL Proteinase stock dissolved in 19.0 mL Tris-EDTA buffer</li> </ul>
<p><b>PIER procedure</b></p> <p>Deparaffinize and rehydrate sections (see chapter <i>Immunostaining with paraffin embedded tissue sections</i>):</p> <ul style="list-style-type: none"> <li>– Proteinase K working solution                      10-20 min at 37°C (optimal enzyme concentrations and incubation times depend on tissue specimen)</li> <li>– cool down to room temperature                      10 min</li> <li>– distilled water    several rinses</li> <li>– PBS or PBS/BSA (optionally)                      3 x 2 min</li> </ul> <p>Perform immunostaining according to standard protocol</p>	

### Trypsin method

Reagents	Solutions
<p>Trypsin Type II from porcine pancreas (Sigma Chemical Co.)</p> <p>Calcium chloride</p> <p>NaOH (1 M)</p> <p>Phosphate buffered saline (PBS)</p> <p>PBS supplemented with 1% BSA (PBS/BSA)</p> <p>PBS supplemented with 1% gelatin</p> <p>Distilled water</p>	<ul style="list-style-type: none"> <li>• 0.5% Trypsin stock solution: 50.0 mg Trypsin dissolved in 10.0 mL distilled water (store at -20°C)</li> <li>• 1% Calcium chloride stock solution: 100.0 mg calcium chloride dissolved in 10.0 mL distilled water (store at 4°C)</li> <li>• Trypsin working solution (0.05%): 1.0 mL Trypsin stock <i>plus</i> 1.0 mL calcium chloride stock dissolved in 8.0 mL distilled water  adjust pH to 7.8 with NaOH and use the solution immediately</li> <li>• Trypsin working solution (0.025%): 0.5 mL Trypsin stock <i>plus</i> 1.0 mL calcium chloride stock dissolved in 8.5 mL distilled water  adjust pH to 7.8 with NaOH and use the solution immediately</li> </ul>
<p><b>PIER procedure</b></p> <p>Deparaffinize and rehydrate sections (see chapter <i>Immunostaining with paraffin embedded tissue sections</i>):</p> <ul style="list-style-type: none"> <li>– Trypsin solution (0.025 or 0.05%)                      10-20 min at 37°C (optimal enzyme concentrations and incubation times depend on tissue specimen)</li> <li>– cool down to room temperature                      10 min</li> </ul>	

– distilled water	several rinses
– PBS/gelatin or PBS/BSA (optionally)	3 x 2 min
Perform immunostaining according to standard protocol	

## Antigen unmasking by heat-induced epitope retrieval (HIER)

About 15 years after the introduction of immunoperoxidase labeling in our routine histology (KUHLMANN WD, 1975; WURSTER K et al, 1978), the invention of heat-induced epitope retrieval techniques by SR SHI et al. (1991) marked a breakthrough in all histopathological laboratories worldwide inasmuch as the detection of many antigens became significantly facilitated which were hitherto difficult to be stained. With the experience over many years it was found that heat mediated epitope retrieval methods are more effective and produce more consistent results than proteolytic digestion methods. Different heating methods have been used and compared in the meantime. Microwave ovens, pressure cookers and steamers are the most often applied devices, but autoclaves and water baths can be employed as well.

Among other effects, it is assumed that HIER will break molecular cross-links and reverse the protein-conformational changes which are induced by fixatives. Many aspects of HIER have been described (SHI SR et al., 1995; TAYLOR CR et al., 1996; SHI SR et al., 1997; SHI SR et al., 1998; MILLER RT et al., 2000; SHI SR et al., 2001; LEONG AS et al., 2002; SOMPURAM SR et al., 2004). These authors found that apart from the application of heat, conditions such as the achieved temperature level, its duration and the type of retrieval buffers greatly influence the unmasking efficiency. However, it must be kept in mind that the degree of fixation will significantly influence the effect of antigen retrieval by heat inasmuch as weakly fixed proteins (in aldehydes) are denatured rapidly at high temperatures, whereas such molecules will not be denatured at high temperatures when they are well fixed. This may explain why tissues preserved in other fixatives than aldehydes can give unsatisfactory immunostaining following epitope retrieval by HIER methods.

So far, temperature has the most important effect on antigen unmasking. The chemicals used in the retrieval solutions including the type of ions, pH and molarity are not essential factors but may be co-factors (reviewed by SHI SR et al., 1997). Calcium chelating agents can be active in heat retrievals as supposed by JM MORGAN et al. (1997). The beneficial effect of calcium chelating agents (EDTA) in retrieval fluids is probably due to reversal of strong bonds between proteins and calcium ions induced by formaldehyde. In this way, EDTA and HIER can reverse conformation changes. Yet, it must be kept in mind that different antigens will behave differently.

The retrieval solutions applied for HIER techniques cover a wide range of pH values. Many studies have confirmed that none of the until now described retrieval solutions can be regarded as the ideal solution for all antigens. Usually, retrieval conditions must be optimized. In the beginning of experimentation or when a new antibody is used, several experiments are needed in order to choose the appropriate reaction conditions for antigen retrieval (AR) by the use of solutions (a) at different pH values, and, (b) at different temperatures. In any case, the combined use of positive and negative reacting tissue sections is needed to establish an optimal protocol. To this end, tissue micro arrays are especially useful.

Some popular retrieval solutions together with the use of different heating devices are summarized in the following Table.

Retrieval method	AR solution pH 1-2 <sup>1</sup>	AR solution pH 6-8 <sup>2</sup>	AR solution pH 9-10 <sup>3</sup>
<b>Water bath (80-95°C)</b>	Procedure: a) no heat b) room temperature for 10-20 min	Procedure: a) 80-95°C, 20-45 min b) cool down to room temperature for 20 min	Procedure: a) 80-95°C, 20-30 min b) cool down to room temperature for 20 min
<b>Steamer (100°C)</b>	Procedure: a) no heat b) room temperature for 10-20 min	Procedure: a) 95-100°C, 10-45 min b) cool down to room temperature for 20 min	Procedure: a) 95-100°C, 10-30 min b) cool down to room temperature for 20 min
<b>Microwave (100°C)</b>	Procedure: a) no heat b) room temperature for 10-20 min	Procedure: a) microwave 2-3 x 5 min at 700-800 Watts b) cool down to room temperature for 20 min	Procedure: a) microwave 2-3 x 5 min at 700-800 Watts b) cool down to room temperature for 20 min
<b>Pressure cooker (120-125°C)</b>	Procedure: a) no heat b) room temperature for 10-20 min	Procedure: a) heat to reach operating temperature and pressure at 120-125°C, continue for 1-10 min b) depressurize for about 10 min c) cool down to room temperature for 10 min	Procedure: a) heat to reach operating temperature and pressure at 120-125°C, continue for 1-10 min b) depressurize for about 10 min c) cool down to room temperature for 10 min

Retrieval methods and antigen retrieval solutions (AR)

<sup>1</sup> Hydrochloric acid in distilled water (pH 0.6-0.9); formic acid (pH 1.6-2.0) and no heat applied for antigen retrieval

<sup>2</sup> Sodium citrate buffer (pH 6.0); citrate-EDTA buffer (pH 6.2); EDTA buffer (pH 8.0)

<sup>3</sup> Tris-EDTA buffer (pH 9.0); Tris-saline buffer (pH 9.0)

Use standardized conditions, e.g. constant buffer volume, same number of slides in each run, starting temperature of the retrieval solutions

## Selection of antigen retrieval solutions

Widely employed buffer solutions for heat-induced antigen unmasking.

### Citrate buffer pH 6.0 method

Reagents	Solutions
Trisodium citrate HCl (1 M) Tween 20	<ul style="list-style-type: none"> <li>0.01 M Citrate buffer pH 6.0: 2.94 g trisodium citrate dissolved in 1000 mL distilled water</li> </ul>

Phosphate buffered saline (PBS) Distilled water	mix and adjust pH to 6.0 <ul style="list-style-type: none"> <li>Citrate-Tween (0.05%) buffer: 0.5 mL Tween 20 dissolved in 1000 mL 0.01 M citrate buffer</li> </ul>
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### Citrate-EDTA buffer pH 6.2 method

Reagents	Solutions
Citric acid (anhydrous) Disodium dihydrogen ethylenediamine tetraacetate (disodium EDTA) Tween 20 Phosphate buffered saline (PBS) Distilled water	<ul style="list-style-type: none"> <li>0.01 M Citrate-EDTA buffer pH 6.2 (0.01 M citric acid and 0.002 M EDTA): 1.92 g citric acid <i>plus</i> 0.74 g EDTA dissolved in 1000 mL distilled water</li> </ul> <p>mix and adjust pH to 6.2</p> <ul style="list-style-type: none"> <li>Citrate-EDTA-Tween (0.05%) buffer: 0.5 mL Tween 20 dissolved in 1000 mL 0.01 M citrate-EDTA buffer</li> </ul>

### Citraconic anhydride buffer pH 7.4 method

Reagents	Solutions
Citraconic anhydride Phosphate buffered saline (PBS) Distilled water	<ul style="list-style-type: none"> <li>0.05% Citraconic anhydride buffer pH 7.4: 0.5 g citraconic anhydride dissolved in 1000 mL distilled water</li> </ul> <p>mix and adjust pH to 7.4</p>

### EDTA buffer pH 8.0 method

Reagents	Solutions
Disodium dihydrogen ethylenediamine tetraacetate (disodium EDTA) NaOH (1 M) Tween 20 Phosphate buffered saline (PBS) Distilled water	<ul style="list-style-type: none"> <li>0.001 M EDTA buffer pH 8.0: 0.37 g EDTA dissolved in 1000 mL distilled water</li> </ul> <p>mix and adjust pH to 8.0</p> <ul style="list-style-type: none"> <li>EDTA-Tween (0.05%) buffer: 0.5 mL Tween 20 dissolved in 1000 mL 0.001 M EDTA buffer</li> </ul>

### Tris-EDTA buffer pH 9.0 method

Reagents	Solutions

Tris (TRIS Base) Disodium dihydrogen ethylenediamine tetraacetate (disodium EDTA) Tween 20 Phosphate buffered saline (PBS) Distilled water	<ul style="list-style-type: none"> <li>Tris-EDTA buffer pH 9.0 (0.01 M Tris and 0.001 M EDTA): 1.21 g Tris <i>plus</i> 0.37 g EDTA are dissolved in 1000.0 mL distilled water</li> </ul> mix and control pH (solution is usually at pH 9.0) <ul style="list-style-type: none"> <li>Tris-EDTA-Tween (0.05%) buffer: 0.5 mL Tween 20 dissolved in 1000 mL 0.01 M Tris-EDTA buffer</li> </ul>
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### Tris-saline buffer pH 9.0 method

Reagents	Solutions
Tris (TRIS Base) Sodium chloride (NaCl) HCl (1 M) Tween 20 Phosphate buffered saline (PBS) Distilled water	<ul style="list-style-type: none"> <li>Tris-NaCl buffer pH 9.0 (0.05 M Tris and 0.15 M NaCl): 6.05 g Tris <i>plus</i> 8.77 g NaCl are dissolved in 1000.0 mL distilled water</li> </ul> mix and adjust pH to 9.0 <ul style="list-style-type: none"> <li>Tris-NaCl-Tween (0.05%) buffer: 0.5 mL Tween 20 dissolved in 1000 mL 0.01 M Tris-NaCl buffer</li> </ul>

### Heat-induced epitope retrieval (HIER)

Several devices can be used to perform heat-retrieval. Generally, all HIER techniques are based on the heating of tissue specimens mounted on slides in a so-called retrieval solution, followed by a cooling down period, washings and subsequent immunostaining. The main difference between the various HIER methods is the heat source by which retrieval solutions and slides are heated. The method of HIER appears to be simple, but the variety of used instruments make comparisons very difficult. Heat devices can be water baths, microwave ovens, steamers, pressure cookers or autoclaves. Each device will have its advantages and disadvantages. For example, some problems with microwaves (household devices) are inherent with the physical principle, i.e. (a) irradiation produces heat which is not equally distributed within the container of retrieval solution (difficult to control), and (b) irradiation is not equally distributed within the microwave oven.

At least some standardization is needed for consistent results, and this includes a more precise control of heating with maintaining constant temperature at preselected levels. Some experimental and hypothetical approaches in this direction were recently considered (SHI SR et al., 2007). In the meantime, several HIER devices can be purchased. The technical advantages or disadvantages of those systems must be considered. With respect to costs, water baths are still useful as a *poor man's* heating device. The main drawback, however, is certainly the achievable temperature limited to 100°C. In this respect, pressure cookers can be more favorable.

### **Basic schedule of HIER**

Deparaffinize and rehydrate sections (see chapter *Immunostaining with paraffin embedded tissue sections*). Water bath, steamer, pressure cooker or microwave oven may be used for heating. The technique of antigen unmasking with a water bath is described.

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|---|---|
| – Retrieval solution  | preheat water bath with staining jar (e.g. COPLIN, HELLEND AHL or SCHIEFFERDECKER) containing the retrieval solution until 95-100°C are reached |
| – antigen retrieval (f.e. water bath)                                     | place slides in preheated retrieval solution, cover with loose-fitting lid and hold the temperature   |
| – incubation time<br>(optimal incubation time depends on tissue specimen) | 20-40 min at 95-100°C   |
| – cool down to room temperature   | remove staining jar from water bath and allow to cool down for 15-20 min  |
| – distilled water   | several rinses  |
| – PBS   | 3 x 2 min   |

Perform immunostaining according to standard protocol

It is also possible to combine HIER and PIER procedures. Such a strategy can be useful when loss of tissue sections due to high temperature heating will occur. In the first step, a lower temperature is applied as usual (f.e. 80°C in citrate buffer). In the second step, antigen retrieval is completed by enzyme (f.e. trypsin) treatment (FROST AR et al., 2000).

Other reported techniques of epitope retrieval have made use of ultrasonic methods (PORTIANSKY EL and GIMENO EJ, 1996), so-called sonication-induced epitope retrieval. This approach, however, is not widely employed until now. Since comparative studies have resulted in contradictory staining results (BRYNES RK et al., 1997; MILLER RT et al., 2000), the usefulness of ultrasound epitope retrieval needs further evaluation.

### **Selected publications for further readings**

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Full version of citations in chapter *References*.