

## IHC-PARAFFIN PROTOCOL (IHC-P)

Immunohistochemistry (or IHC) is a method for demonstrating the presence and location of proteins in tissue sections. Though less sensitive quantitatively than immunoassays such as Western blotting or ELISA, it enables the observation of processes in the context of intact tissue. This is especially useful for assessing the progression and treatment of diseases such as cancer. In general, the information gained from IHC combined with microscopy literally provides a “big picture” that can help make sense of data obtained using other methods.

Immunohistochemical staining is accomplished with antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is then visualized using either chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate at the location of the protein, or fluorescent detection, in which a fluorophore is conjugated to the antibody and can be visualized using fluorescence microscopy.

IHC-P refers to the staining of tissues that have been fixed (usually in neutral buffered formalin) and then embedded in paraffin before being sectioned. The basic steps of the IHC-P protocol are as follows:

1. Fixing and embedding the tissue
2. Cutting and mounting the section
3. Deparaffinizing and rehydrating the section
4. Antigen retrieval
5. Immunohistochemical staining
6. Counterstaining (if desired)
7. Dehydrating and stabilizing with mounting medium
8. Viewing the staining under the microscope

### A. Optimizing a new antibody for IHC-P

1. Antigen retrieval
2. Primary antibody concentration
3. Detection

### B. Fixation

### C. Deparaffinization

### D. Antigen retrieval

1. Buffer solutions for heat-induced epitope retrieval
2. Heat-induced epitope retrieval methods
3. Enzymatic antigen retrieval

### E. Immunohistochemical staining

1. General guidelines
2. Protocol
3. Controls
4. Signal amplification

### F. Resources

### G. Additional buffer recipes

## A. Optimizing a new antibody for IHC-P

When using a new antibody in IHC-P, the antibody must be tested to find the optimal staining conditions. Each antigen has a preferred method of antigen retrieval, and each antibody has an optimal dilution.

### 1. Antigen retrieval

Try staining without antigen retrieval, and also using the following antigen retrieval methods. Detailed protocols for these procedures are in the Antigen Retrieval section.

Heat-induced: Sodium citrate 10 mM, pH 6.0

Heat-induced: Tris/EDTA pH 9.0

Enzymatic: trypsin, pepsin, or other protease

Once the optimal antigen retrieval method is established, the antibody concentration can be fine-tuned.

### 2. Primary antibody concentration

If the concentration of the antibody is provided, we recommend trying 0.5 µg/ml and 5 µg/ml overnight at 4°C. If the antibody is unpurified, we recommend starting with the following starting dilutions, and also testing a 20-fold higher dilution.

Whole antiserum: 1/50

Ascites: 1/100

Tissue Culture supernatant: undiluted (also referred to as "neat")

### 3. Detection

We recommend horseradish peroxidase (HRP) for visible light microscopy. Peroxide/DAB are the substrate and recommended chromogen for horseradish peroxidase. Various fluorochrome-conjugated antibodies are available for fluorescent microscopy; choice will be dictated by the needs of the experiment.

## B. Fixation

Proper fixation is key for the success of immunohistochemistry. 10% neutral buffered formalin (NBF) is most commonly used. Where Abcam's datasheets state IHC-P as a tested application, this fixative has been used unless stated otherwise. Other fixatives such as paraformaldehyde (PFA) or Bouin solution (formalin/picric acid) are used less frequently.

The ideal fixation time will depend on the size of the tissue block and the type of tissue, but fixation between 18-24 hours seems to be ideal for most applications. Under-fixation can lead to edge staining, with strong signal on the edges of the section and no signal in the middle; over-fixation can mask the epitope. Antigen retrieval can help overcome this masking, but if the tissue has been fixed for a long period of time (i.e. over a weekend), there may be no signal even after antigen retrieval.

After fixation, the tissue block is embedded in paraffin, then cut in a microtome to the desired thickness (approximately 5 microns is ideal for IHC) and affixed onto the slide. Tissue sections are best mounted on positively charged or APES (amino-propyl-tri-ethoxy-silane) coated slides. Once mounted, the slides should be dried to remove any water that may be trapped under the section. This can be done by leaving the slide at room temperature overnight. If there is a problem with the section adhering to the slide, you may also incubate the slide at 60°C for a few hours.

## C. Deparaffinization

Before proceeding with the staining protocol, the slides must be deparaffinized and rehydrated. Incomplete removal of paraffin can cause poor staining of the section.

### Materials and reagents

- Xylene
- 100% ethanol
- 95% ethanol

### Method

Place the slides in a rack, and perform the following washes:

1. Xylene: 2 x 3 minutes
2. Xylene 1:1 with 100% ethanol: 3 minutes
3. 100% ethanol: 2 x 3 minutes
4. 95% ethanol: 3 minutes
5. 70 % ethanol: 3 minutes
6. 50 % ethanol: 3 minutes
7. Running cold tap water to rinse

Keep the slides in the tap water until ready to perform antigen retrieval. At no time from this point onwards should the slides be allowed to dry. Drying out will cause non-specific antibody binding and therefore high background staining.

## D. Antigen retrieval

Most formalin-fixed tissue requires an antigen retrieval step before immunohistochemical staining can proceed. This is due to the formation of methylene bridges during fixation, which cross-link proteins and therefore mask antigenic sites. The two methods of antigen retrieval are heat-mediated (also known as heat-induced epitope retrieval, or HIER) and enzymatic.

Both antigen retrieval methods serve to break the methylene bridges and expose the antigenic sites in order to allow the antibodies to bind. Some antigens prefer enzymatic to heat mediated antigen retrieval and vice versa. Enzymatic retrieval can sometimes damage the morphology of the section, so the concentration and treatment time need to be tested. Antigen retrieval with Tris/EDTA pH 9.0 buffer is suitable for most antigens. Sodium citrate pH 6.0 is also widely used. Please see

[http://www.nordiqc.org/Techniques/Epitope\\_retrieval.htm](http://www.nordiqc.org/Techniques/Epitope_retrieval.htm)

for an explanation of why Abcam recommends using Tris/EDTA pH 9.0 buffer before sodium citrate pH 6.0.

Heat-induced epitope retrieval is most often performed using a pressure cooker, a microwave, or a vegetable steamer. Additionally, some labs will use a water bath set to 60°C and incubate the slides in retrieval solution overnight. Unless the antigen retrieval method is stated on the antibody datasheet, the optimal method for each antigen must be found experimentally. Abcam recommends testing several methods to find the retrieval that gives optimal staining.

## 1. Buffer solutions for heat-induced epitope retrieval

The following solutions are three of the more popular buffers for HIER. In the absence of advice from other researchers for a particular antibody, choice of retrieval buffer is best accomplished by experiment.

- Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)

Tri-sodium citrate (dihydrate)    2.94 g  
 Distilled water                      1000 ml  
 Mix to dissolve. Adjust pH to 6.0 with 1N HCl.  
 Add 0.5 ml of Tween 20 and mix well. Store at room temperature for 3 months or at 4C for longer storage.

- 1 mM EDTA, adjusted to pH 8.0

EDTA                      0.37 g  
 Distilled water 1000 ml  
 Store at room temperature for 3 months.

- Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0)

Tris            1.21 g  
 EDTA        0.37 g  
 Distilled water 1000 ml (100 ml to make 10x, 50 ml to make 20x)  
 Mix to dissolve. pH is usually at 9.0.  
 Add 0.5 ml of Tween 20 and mix well. Store at room temperature for 3 months or at 4C for longer storage.

## 2. Heat-induced epitope retrieval methods

### a) Pressure cooker

Slides should be placed in a metal rack for this procedure.

#### Materials and reagents

- Domestic stainless steel pressure cooker
- Hot plate
- Vessel with slide rack to hold approximately 400-500 ml
- Antigen retrieval buffer (i.e. Tris/EDTA pH 9.0, sodium citrate pH 6.0)

#### Method

- Add the appropriate antigen retrieval buffer to the pressure cooker. Place the pressure cooker on the hotplate and turn it on full power. Do not secure the lid of the pressure cooker at this point, simply rest it on top.  
While waiting for the pressure cooker to come to a boil, deparaffinize and rehydrate the sections as above.
- Once boiling, transfer the slides from the tap water to the pressure cooker. **USE CARE WITH HOT SOLUTION - USE FORCEPS!** Secure the pressure cooker lid as in the manufacturer's instructions.
- As soon as the cooker has reached full pressure (see the manufacturers instructions), time 3 minutes (See note i).
- When 3 minutes has elapsed, turn off the hotplate and place the pressure cooker in an empty sink.
- Activate the pressure release valve (see the manufacturer's instructions) and run cold water over the cooker. Once de-pressurized, open the lid and run cold water into the cooker for 10 minutes. **USE CARE WITH HOT SOLUTION!** (See note ii).
- Continue with the immunohistochemical staining protocol.

## Notes and tips



- i. Three minutes is only suggested as a starting point antigen retrieval time. Less than 3 minutes may leave the antigens under-retrieved, leading to weak staining. More than 3 minutes may leave them over-retrieved, leading to non-specific background staining and also increasing the chances of sections dissociating from the slides. A control experiment is recommended beforehand, where slides of the same tissue section are retrieved for 1, 2, 3, 4 and 5 minutes before being immunohistochemically stained to evaluate optimum antigen retrieval time for the particular antibody being used.
- ii. This is to allow the slides to cool enough so they may be handled, and to allow the antigenic site to re-form after being exposed to such high temperature

## b) Microwave

The use of a domestic microwave is inadvisable. Hot and cold spots are common, leading to uneven antigen retrieval. Antigen retrieval times are usually longer, due to the absence of a pressurized environment, nearly always leading to section dissociation. A scientific microwave is much more appropriate. Most brands have on-board pressurized vessels and can keep the temperature at a constant 98°C to avoid section dissociation. The only drawback is the expense of purchasing one!

When using this method, it is possible for the buffer to boil over, and a large amount of the retrieval buffer will evaporate. Be sure to watch the buffer level of the slide vessel, and add more buffer if necessary. Do not allow the slides to dry out.

*Slides should be placed in a plastic rack and vessel for this procedure. Standard glass histology staining racks and vessels will crack when heated.*



## Materials and reagents

- Domestic (850W) or scientific microwave
- Microwaveable vessel with slide rack to hold approximately 400-500 ml or Coplin jar
- Antigen retrieval buffer (e.g. Tris/EDTA pH 9.0, sodium citrate pH 6.0, etc.)

## Method

1. Deparaffinize and rehydrate the sections as above.
2. Add the appropriate antigen retrieval buffer to the microwaveable vessel (See note i).
3. Remove the slides from the tap water and place them in the microwaveable vessel. Place the vessel inside the microwave. If using a domestic microwave, set to full power and wait until the solution comes to the boil. Boil for 20 minutes from this point. If using a scientific microwave, program so that antigens are retrieved for 20 minutes once the temperature has reached 98°C. (See note ii).
4. When 20 minutes has elapsed, remove the vessel and run cold tap water into it for 10 minutes. Use care with hot solution. (See note iii).
5. Continue with the immunohistochemical staining protocol.

## Notes

- i. Use a sufficient volume of antigen retrieval solution in order to cover the slides by at least a few centimeters if using a non-sealed vessel to allow for evaporation during the boil. Be sure to watch for evaporation and for boiling over during the procedure, and do not allow the slides to dry out!
- ii. 20 minutes is only a suggested antigen retrieval time. Less than 20 minutes may leave the antigens under-retrieved, leading to weak staining. More than 20 minutes may leave them over-retrieved, leading to non-specific background staining and also increasing the chances of sections dissociating from the slides. A control experiment is recommended beforehand, where slides of the same tissue section are retrieved for 5, 10, 15, 20, 25 and 30 minutes before being immunohistochemically stained to evaluate optimum antigen retrieval time for the particular antibody being used.
- iii. This allows the slides to cool enough so they may be handled, and allows the antigenic site to re-form after being exposed to high temperature.

### c) Vegetable steamer

Many labs use a vegetable steamer or rice cooker for heat-mediated antigen retrieval. The procedure is similar to microwaving in that it maintains the temperature of the buffer at 100 °C, but without the vigorous boiling of the microwave method. This method may be adapted to a water bath set at 100 °C in place of the steamer.

*Slides should be placed in a plastic or metal rack and vessel for this procedure. Standard glass histology staining racks and vessels will crack when heated.*



#### Materials and reagents

- Vegetable steamer
- Vessel with slide rack to hold approximately 400-500 ml (or 250 ml if using Tissue –Tek containers)
- Antigen retrieval buffer (e.g. Tris/EDTA pH 9.0, sodium citrate pH 6.0, etc.)

#### Method

1. Deparaffinize and rehydrate the sections as above.
2. Set up the vegetable steamer according to the manufacturer's instructions and preheat.
3. Pre-heat the appropriate antigen retrieval buffer to boiling in a flask (a microwave is handy for this).
4. Put the container that will hold the rack of slides into the vegetable steamer.
5. Carefully add the hot buffer to the container, followed by the rack of slides. If more convenient, add the buffer to the container before placing the container in the steamer.
6. Close the lid of the steamer. The container of buffer should also have a lid. The rack of slides will initially bring the temperature of the AR solution down but it will return to 95 - 100 °C within several minutes.
7. Keep the container in the steamer for 20 minutes from this point. (See note ii for the microwave method).
8. When 20 minutes has elapsed, remove the vessel and run cold tap water into it for 10 minutes. Use care with hot solution. (See note iii for the microwave method).
9. Continue with the immunohistochemical staining protocol.

### 3. Enzymatic antigen retrieval

Choice of enzyme will be indicated on the datasheet for the antibody. If not, trypsin has been shown to be useful for a wide range of antigens that require retrieval post-formalin/PFA fixation.

There are at least two methods for applying the enzyme solution to the tissue: directly pipetting the solution onto the tissue on the slide, or placing a rack of tissue slides into a container of enzyme solution. The first method uses less reagent but since each slide needs to be handled individually, the incubation time needs to be monitored carefully for each slide to ensure all slides are receiving the same treatment. For this reason, it is easier to treat large batches of slides (e.g. > 5) by immersing them in a container of enzyme solution. If using an automated staining system (e.g. Ventana), consult the manufacturer for an appropriate enzymatic retrieval protocol.

#### a) Enzymatic retrieval, pipetting method

##### Materials and reagents

- 37 °C incubator
- Humidified chamber (either the incubator itself or a container with a wet paper towel)
- Two slide rack containers of TBS with slide rack (See section G for TBS recipe.)
- Enzymatic antigen retrieval solution (For trypsin, see below. For pepsin and proteinase K, see Section G.)

The following method uses trypsin. There are commercially available trypsin preparations optimized for IHC (Abcam has a convenient trypsin product, catalogue ID ab970), or it can be prepared as follows:

- Trypsin Stock Solution (0.5% in distilled water)

Trypsin	50 mg
Distilled water	10 ml
Mix to dissolve. Store at -20 °C.	

- Calcium Chloride Stock Solution (1%)

Calcium chloride            0.1 g  
Distilled water            10 ml  
Mix well and store at 4 °C.

- Trypsin Working Solution (0.05%)

Trypsin stock solution (0.5%)            1 ml  
Calcium chloride stock solution 1%    1 ml  
Distilled Water                            8 ml  
Adjust pH to 7.8 with 1N NaOH. Store at 4 °C for one month or -20 °C for long term storage.

## Method

1. Prepare the trypsin and pre-heat to 37°C. Carefully blot excess water from the around the tissue section and pipet the enzyme solution (generally 50 - 100 ul will suffice) onto the section. It may be necessary to spread the solution around the section with the pipet tip; be careful not to damage the tissue.
2. Place the slides in a humidified container and then into the 37°C incubator. Avoid placing the slides directly on the incubator shelves as there will be variations in temperature that could affect staining intensity. Ideally, the container holding the slides is pre-heated in the incubator.
3. After 10- 20 minutes (this will need to be optimized), remove the slides from the incubator and transfer to a rack in a container of tap water. Rinse by running tap water for 3 minutes.
4. Continue with immunohistochemical staining protocol.

## b) Enzymatic retrieval, immersion method

### Materials and reagents

- 37°C waterbath
- Slide racks and slide rack containers
- Enzymatic antigen retrieval solution (For trypsin, see pipetting method. For pepsin and proteinase K, see Section G.)

## Method

1. Set water bath to the optimal temperature for the enzyme you are using. Add ultrapure water to two containers that can hold slide racks. Place the containers into the water bath to warm. (See note ii).
2. Deparaffinize and rehydrate sections as above. Place slides in one water container to warm (See note iii).
3. Prepare the enzymatic antigen retrieval buffer from the warm water in the other container, and then return the container to the water bath to allow the solution to re-heat (See note iv).
4. Transfer the warmed slides into the enzyme solution for 10 - 20 minutes (see note v.) with intermittent gentle agitation, then remove the slides and place them in running tap water for 3 minutes to rinse off the enzyme.
5. Continue with immunohistochemical staining protocol.

## Notes

- i. Be sure to read the manufacturer's literature for the enzyme you choose, as some enzymes require specific buffers and cofactors for activity.
- ii. Use a sufficient volume of water or buffer to cover the slides.
- iii. Placing cold slides into the enzyme solution will lower the temperature of the solution, reducing enzyme activity and leading to under-retrieval of the antigenic site.
- iv. Prepare the enzymatic antigen retrieval solution as quickly as possible to avoid impairing the activity of the enzyme. Allow this solution to return to temperature before introducing the slides.
- v. Ten to twenty minutes is only suggested as a starting point incubation time. Less than 10 minutes may leave the antigens under retrieved, leading to weak staining. More than 20 minutes may leave them over



retrieved, leading to non-specific background staining and also increasing the chances of sections dissociating from the slides or damage to the morphology of the tissue. A control experiment is recommended beforehand, where slides of the same tissue section are incubated in the enzyme solution for 10, 15, 20, 25, and 30 minutes before being immunohistochemically stained to evaluate optimum antigen retrieval time for the particular antibody being used.

## E. Immunohistochemical staining

### 1. General guidelines

The following protocol assumes the laboratory does not have an automated stainer or other capillary gap system that allows rapid application and rinsing of reagents (e.g. Shandon Sequenza). Reagents can be applied manually by pipet or the sequence of the protocol can be adapted to automated and semi-automated systems if these are available.

All incubations should be carried out in a humidified chamber to avoid drying of the tissue. Drying at any stage will lead to non-specific binding and ultimately high background staining. A shallow, plastic box with a sealed lid and wet tissue paper in the bottom is an adequate chamber, just as long as the slides are kept off the paper and can lay flat so that the reagents don't drain off! A good solution is to cut a plastic serological pipette into lengths to fit your incubation chamber. Glue them in pairs to the bottom of the chamber, with the 2 individual pipette tubes of each pair being placed about 4.0 cm apart. This provides a level and raised surface for the slides to rest on away from the wet tissue paper.

Dilutions of the primary and secondary antibody are listed on the datasheets or are determined by testing a range. Adjust dilutions appropriately from the results obtained. Adhere strictly to all incubation times in the protocol.

For enzymatic methods, horseradish peroxidase (HRP) or alkaline phosphatase (AP) are the most commonly used enzymes. There are a number of chromogens used with these enzymes (see note x).

### 2. Protocol

Please refer to Notes section for the theory and section G for buffer recipes.  
If necessary, perform antigen retrieval before commencing with the following protocols.

#### Day 1

1. (If using an HRP conjugate for detection, blocking of endogenous peroxidase can be performed here but we recommend waiting until after the primary antibody incubation. See Day 2, step 2 and note v.).
2. Wash the slides 2 x 5 minutes in TBS plus 0.025% Triton X-100 with gentle agitation. (See note i).
3. Block in 10% normal serum with 1% BSA in TBS for 2 hours at room temperature (See note iii).
4. Drain slides for a few seconds (do not rinse) and wipe around the sections with tissue paper (See note iv).
5. Apply primary antibody diluted in TBS with 1% BSA (See note v).
6. Incubate overnight at 4°C (See note iv).

#### Day 2

1. Rinse 2 x 5min TBS 0.025% Triton with gentle agitation.
2. If using an HRP conjugate for detection, incubate the slides in 0.3% H<sub>2</sub>O<sub>2</sub> in TBS for 15 min (See note v.).
3. For enzymatic detection (HRP or AP secondary conjugates):

Apply enzyme-conjugated secondary antibody to the slide diluted to the concentration recommended by the manufacturer in TBS with 1% BSA, and incubate for 1 hour at room temperature.

For fluorescent detection:



Apply fluorophore-conjugated secondary antibody to the slide diluted to the concentration recommended by the manufacturer in TBS with 1% BSA, and incubate for 1 hour at room temperature.

*This step should be done in the dark to avoid photobleaching.*



4. Rinse 3 x 5min TBS.  
If using fluorescent detection, end at this step and coverslip with mounting medium.  
If visualizing the protein with a chromogen, continue with the following steps.
5. Develop with chromogen for 10 min at room temperature (See note vi.).
6. Rinse in running tap water for 5 min.
7. Counterstain (if required) (See note vii.).
8. Dehydrate, clear and mount (See notes ix. and x.).

### 3. Controls

To estimate the contribution of the non-specific interaction and Fc receptor binding, staining protocols using an antibody directed to an irrelevant antigen (for example, BrdU) having the same isotype as the antibody of interest may be analyzed in parallel with the antibody of interest. The antibody directed to the irrelevant antigen is known as the isotype control. For whole serum antibodies, use normal serum from an unimmunized animal of the same species as the primary antibody.

If an isotype control is not available, a negative antibody control is recommended. Simply replace the primary antibody with antibody diluent.

A positive tissue control is strongly recommended to ensure that the antibody is performing as expected. Depending on the experiment, it may also be useful to include a negative tissue control: a tissue in which the protein of interest is not expected to be found.

### Notes and Tips:



- i. The use of 0.025% Triton X-100 in the TBS helps to reduce surface tension, allowing reagents to cover the whole tissue section with ease. It is also believed to dissolve Fc receptors, therefore reducing non-specific binding. Abcam recommends TBS to give a cleaner background than PBS.
- ii. The secondary antibody may cross react with endogenous immunoglobulins in the tissue. This is minimized by pre-treating the tissue with normal serum from the species in which the secondary was raised. The use of normal serum before the application of the primary also eliminates Fc receptor binding of both the primary and secondary antibody. BSA is included to reduce non-specific binding caused by hydrophobic interactions.
- iii. The primary antibody should be diluted to the manufacturer's recommendations or to a previously optimized dilution. If there is no suggested starting point, we recommend following the recommendations in Section A. Most antibodies will be used in IHC-P at a concentration between 0.5 and 10 µg/ml.

Make sure the primary antibody is raised in a species different from the tissue being stained. If, for example, you had mouse tissue and your primary antibody was raised in a mouse, an anti-mouse IgG secondary antibody would bind to all the endogenous IgG in the mouse tissue, leading to high background. Use of mouse monoclonals on mouse tissue is discussed in our mouse-on-mouse protocol.

- iv. Overnight incubation allows antibodies of lower titer or affinity to be used by simply allowing more time for the antibodies to bind. Also, regardless of the antibody's titer or affinity for its target, once the tissue has reached saturation point no more binding can take place. Overnight incubation ensures that this occurs.
- v.  $H_2O_2$  suppresses endogenous peroxidase activity and therefore reduces background staining. To check for the presence of endogenous peroxidases, incubate a tissue slide after re-hydration in a solution of DAB. If areas of the section appear brown under the microscope, a blocking step should help reduce this staining. Some epitopes are modified by peroxide, leading to reduced antibody:antigen binding. Incubating sections with peroxide after the primary incubation avoids this problem.

Peroxide can be diluted in TBS or water. Some laboratories use methanol which is useful for blood smears or other peroxidase-rich tissues; peroxide diluted in methanol tends to reduce damage to the tissue caused by the reaction in aqueous solutions. For other tissue though, we recommend diluting in TBS or water. Reduced binding of some antibody:antigen pairs, in particular cell surface proteins, has been observed after methanol/peroxide incubation.

(If using AP or fluorescent detection, omit peroxidase quenching as it only applies to HRP conjugates).

- vi. Develop the colored product of the enzyme with the appropriate chromogen. The choice depends on which enzyme label you are using, the colored end product you prefer and whether you are using aqueous or organic mounting media (See note xii for further details). Some commonly used substrates are listed below:

Enzyme	Substrate	Color	Advantages	Disadvantages	Abcam ID
Horseradish peroxidase (HRP)	3,3'-Diaminobenzidine (DAB)	Brown	Intense color; permanent	Endogenous peroxidase activity in the tissue can lead to false positive staining. AEC is alcohol soluble and incompatible with organic mounting media.	Ab5814 Ab675 Ab8584
	3-Amino-9-ethyl carbazol (AEC)	Red	Intense color; contrasts well with blue for double staining		
Alkaline phosphatase (AP)	5-bromo-4-chloro-3-indoyl phosphate; Nitroblue tetrazolium (BCIP/NBT)	Blue	Intense color	Endogenous alkaline phosphatase activity in the tissue can lead to false positive staining.	Ab8589 Ab7413 Ab7468
	Vector Blue	Blue	Less intense color, but better for double staining		
Glucose oxidase	Nitroblue tetrazolium (NBT)	Blue	No endogenous enzyme activity	Low staining intensity (high concentration of primary and secondary antibodies required for effectiveness)	

- vii. Some commonly used counterstains are hematoxylin (blue), nuclear fast red, or methyl green. When using fluorescent detection, DAPI (blue) or propidium iodide/PI (red) can be used to counterstain.
- viii. Don't forget that DAB is a suspected carcinogen. Wear the appropriate protective clothing. Deactivate it with chlorox in a sealed container overnight (it produces noxious fumes when chlorox is added) and dispose of it according to laboratory guidelines.

If using AP, add 0.24 mg/ml Levamisole (Sigma L9756) to the chromogen solution. Levamisole suppresses endogenous phosphatase activity and therefore reduces background staining.

- ix. If using AEC, Fast Red, INT or any other aqueous chromogen then don't forget that they are alcohol soluble. Use a suitable aqueous mounting media. Don't dehydrate and clear!
- x. Dehydrate and clear DAB, New Fuchsin, Vega Red, NBT, TNBT or any other organic chromogen developed sections by sending them through the rehydration protocol listed in section C in the opposite order. Mount sections in a suitable organic mounting media. Sections mounted in organic mounting media have a better refractive index than those mounted in aqueous mounting media. This means that the image seen down the microscope will be sharper and clearer if organic mounting media is used.

#### **4. Signal amplification**

To achieve a stronger signal, various strategies have been developed to add more enzyme or fluorophore to the target of interest.

##### **a) Avidin-biotin complex (ABC)**

This technique, developed by Su-Ming Hsu and colleagues (J Histochem Cytochem. 1981 Apr 29 (4):577-80), utilizes the high affinity of avidin, a protein found in chicken egg white, for biotin, an enzyme co-factor in carboxylation reactions. Avidin has four binding sites for biotin and binding is essentially irreversible.

In brief, the primary antibody is bound to the protein of interest. A biotinylated secondary antibody is then bound to the primary antibody. In a separate reaction, a complex of avidin and biotinylated enzyme is formed by mixing the two in a ratio that leaves some of the binding sites on avidin unoccupied. This complex is then incubated with the tissue section after the antibody incubations. The unoccupied biotin-binding sites on the complex bind to the biotinylated secondary antibody. The result is more enzyme attached to the target than is possible using an enzyme-conjugated secondary or primary antibody.

The components of the avidin-biotin complex are commercially available in kits that provides the two reagents and instructions for combining them in the optimal ratio. The complex can be used with any of Abcam's biotinylated antibodies. One concern is the presence of endogenous biotin in tissues such as kidney, liver, brain, prostate, colon, intestines, and testes, which can bind the avidin-biotin complex leading to background staining. (Wang and Pevsner, Cell Tissue Res. 1999 Jun;296(3):511-6.) To block binding to endogenous biotin, Abcam offers a blocking kit, ab3387.

##### **b) Labeled streptavidin biotin (LSAB)**

This method is similar to ABC in that it utilizes the interaction of streptavidin (similar to avidin in binding affinity) and biotin. The primary antibody is followed by a biotinylated anti-Ig secondary antibody, followed by streptavidin conjugated to an enzyme or fluorophore. Abcam offers a streptavidin – HRP conjugate, ab7403.

Streptavidin produces less non-specific background staining than avidin since it is non-glycosylated (unlike avidin), and so shows no interaction with lectins or other carbohydrate-binding proteins. For a comparison to ABC in which LSAB was shown to be 4 to 8 times more sensitive, see Giorno R, Diagn Immunol. 1984;2(3):161-6.

##### **c) HRP polymer**

Both avidin-biotin methods (ABC and LSAB) are losing favor to new polymer-enzyme-antibody products that consist of a secondary antibody (e.g. anti-mouse and/or rabbit IgG) attached to a polymer-enzyme complex. One step is eliminated compared to the AB methods and the issue of endogenous biotin is avoided. Abcam offers ab2891, a goat anti-rabbit/mouse IgG HRP polymer.

#### d) Tyramide signal enhancing (TSE)

One of the most effective amplification procedures is the patented and licensed method, TSE (also known as TSA or CSA, depending on the manufacturer of the commercially available kits). It is particularly useful for detection of relatively sparse antigens that other systems have difficulty detecting, and for improving results obtained with poorly-performing antibodies.

The method relies on a peroxidase-catalyzed reaction to covalently attach the tyramide portion of tyramine-protein conjugates to the area around the protein of interest, after first applying a primary antibody and secondary-HRP conjugate. The covalently attached protein cannot be washed off, even if the slides are treated to remove the antibodies, since the tyramide bond is covalent. To obtain a signal, an antibody-enzyme or -fluorophore conjugate is directed against the protein portion of the tyramine-protein conjugate. In one commercially available version of the method, the protein is biotin and a streptavidin-enzyme conjugate is applied instead of an antibody-conjugate. The disadvantages of the procedure are the expense of the kits and the time required to perform the multiple steps.

## F. Resources

IHC World ([www.ihcworld.com](http://www.ihcworld.com)) has a wealth of information on antigen retrieval, positive controls, and troubleshooting the immunohistochemical procedure.

Histonet ([www.histosearch.com/histonet.html](http://www.histosearch.com/histonet.html)) searches archived messages posted to the Histonet listserver from scientists around the world.

Nordic immunohistochemical Quality Control ([www.nordiqc.org](http://www.nordiqc.org)) has information regarding appropriate positive controls and antigen retrieval steps for many target proteins.

## G. Buffer recipes

### Fixation:

#### Formalin Solution (10%, buffered neutral):

Formaldehyde (37-40%) \_\_\_\_ 100 ml  
 Distilled water \_\_\_\_ 900 ml  
 NaH<sub>2</sub>PO<sub>4</sub> \_\_\_\_ 4.0 g  
 Na<sub>2</sub>HPO<sub>4</sub> (anhydrous) \_\_\_\_ 6.5 g

Mix to dissolve.

#### Paraformaldehyde (4%, buffered neutral):

##### 8% Paraformaldehyde:

Paraformaldehyde \_\_\_\_ 40 g  
 Distilled water \_\_\_\_ 500 ml

##### 0.2M Phosphate Buffer (PB), pH 7.4:

Na<sub>2</sub>HPO<sub>4</sub> \_\_\_\_ 10.9 g  
 NaH<sub>2</sub>PO<sub>4</sub> \_\_\_\_ 3.2 g  
 Distilled water \_\_\_\_ 500 ml

The solution should be at pH 7.4. Do not pH using acid or base! If you need to adjust the pH, make up a separate 0.2M solution of either the monobasic or dibasic sodium phosphate (depending on how you need to adjust the pH) and add accordingly.

Heat 8% PFA solution at 60°C while stirring (do not allow the solution to go above 60°C). Once the solution has reached 60°C and the PFA is dissolved, add 500 ml of 0.2M phosphate buffer, to bring the solution to 4% PFA in 0.1M phosphate. Carefully add 1N NaOH dropwise until solution is clear (try 1-2 drops per 500 ml; if still not clear, add a few more drops. Alternatively, you can add 2 pellets of solid NaOH in 1-2L of solution). Cool the solution and filter.

*PFA should always be made up fresh on the same day you wish to use it. Storage overnight at 4°C is possible, but it will not fix as well the second day. It is possible possible to freeze the PFA solution at -20 °C, but for consistency of results, tissue should either be fixed always with fresh PFA or always with freshly thawed PFA.*



Bouin Solution (especially for preserving soft and delicate structures such as brain tissues)

Picric acid (saturated) \_\_\_\_\_ 75 ml  
 Formaldehyde (37-40%) \_\_\_\_\_ 25 ml  
 Glacial acetic acid \_\_\_\_\_ 5 ml

Mix well.