

IHC antigen retrieval protocol

Heat induced epitope retrieval and
enzymatic retrieval

Introduction

Most formalin-fixed tissues require an antigen retrieval step before immunohistochemical staining. Methylene bridges formed during fixation cross-link proteins and mask antigenic sites. Antigen retrieval methods break these methylene bridges and expose antigenic sites, allowing antibodies to bind.

The two methods for antigen retrieval are heat induced epitope retrieval (HIER) and enzymatic retrieval.

Enzymatic retrieval can sometimes damage the morphology of the section, so the concentration and treatment time need to be tested.

Heat-induced epitope retrieval is most often performed using a pressure cooker, a microwave, or a vegetable steamer. Some labs use a water bath set to 60°C and incubate the slides in retrieval solution overnight. This is useful when working with tissue sections that fall off the slide when heated at higher temperatures; in particular bone, cartilage, and skin.

Unless the antigen retrieval method is stated on the antibody datasheet, the optimal method for each antigen must be found experimentally. This applies also to the choice of buffer used for heat-mediated retrieval. The most commonly used buffers are 10 mM sodium citrate pH 6, Tris-EDTA pH 9, and EDTA pH 8. We recommend testing several methods to find the retrieval method that gives optimal staining.

Buffer solutions for heat-induced epitope retrieval

The following solutions are three of the more popular buffers for HIER. In the absence of datasheet information, choice of retrieval buffer is best accomplished by trial.

Sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0)

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

1 mM EDTA, pH 8.0

- EDTA 0.37 g
- Distilled water 1 L
- Adjust to pH 8.0 with NaOH
- Store at room temperature for 3 months

Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0)

- Tris 1.21 g
- EDTA 0.37 g
- Distilled water 1 L
- Mix to dissolve. Adjust pH to 9.0.
- Add 0.5 mL of Tween 20 and mix well. Store at room temperature for 3 months or at 4°C for longer storage.

Heat-induced epitope retrieval methods

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 (Tris/EDTA pH 9.0, sodium citrate pH 6.0, or other)

Method:

A control experiment is recommended to optimize retrieval time, where slides of the same tissue section are retrieved for 1, 2, 3, 4 and 5 min before being immunohistochemically stained.

1. 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, de-paraffinize and rehydrate the sections.
2. Once boiling, transfer the slides from the tap water to the pressure cooker.

Use care with hot solution – use forceps.

3. Secure the pressure cooker lid as per the manufacturer's instructions.
4. As soon as the cooker has reached full pressure, time 3 min.
5. When 3 min have elapsed, turn off the hotplate and place the pressure cooker in an empty sink.
6. Activate the pressure release valve and run cold water over the cooker. Once de-pressurized, open the lid and run cold water into the cooker for 10 min.

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 high temperature.

Handle hot solutions with care.

7. Continue with the immunohistochemical staining protocol.

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 more appropriate. Most brands have onboard pressurized vessels and can keep the temperature at a constant 98°C to avoid section dissociation.

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:

- Scientific microwave or domestic (850 W)
- Microwaveable vessel with slide rack to hold approximately 400–500 mL or Coplins jar
- Antigen retrieval buffer (eg Tris/EDTA pH 9.0, sodium citrate pH 6.0, etc.)

Method:

1. Deparaffinize and rehydrate the sections.

Use a sufficient volume of antigen retrieval solution to cover the slides by at least a few centimeters.

2. Add the appropriate antigen retrieval buffer to the microwaveable vessel.

Use a non-sealed vessel to allow for evaporation during the boil. Be sure to monitor for evaporation and watch out for boiling over during the procedure and do not allow the slides to dry out.

3. Place the slides 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 min from this point. If using a scientific microwave, program so that antigens are retrieved for 20 min once the temperature has reached 98°C.

Use a non-sealed vessel to allow for evaporation during the boil. Be sure to monitor for evaporation and watch out for boiling over during the procedure and do not allow the slides to dry out.

4. When 20 min has elapsed, remove the vessel and run cold tap water into it for 10 min. Use care with hot solution.

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.

5. Continue with the immunohistochemical staining protocol.

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 (eg Tris/EDTA pH 9.0, sodium citrate pH 6.0)

Method:

1. Deparaffinize and rehydrate the sections as above.
2. Set up the vegetable steamer according to the manufacturer's instructions and preheat.

Use a non-sealed vessel to allow for evaporation during the boil. Be sure to monitor for evaporation and watch out for boiling over during the procedure and do not allow the slides to dry out.

3. Pre-heat the appropriate antigen retrieval buffer to boiling in a flask.
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 antigen retrieval solution down but it will return to 95–100°C within several min.
7. Keep the container in the steamer for 20 min from this point.
8. When 20 min has elapsed, remove the vessel and run cold tap water into it for 10 min. Use care with hot solution.

Use a non-sealed vessel to allow for evaporation during the boil. Be sure to monitor for evaporation and watch out for boiling over during the procedure and do not allow the slides to dry out.

9. Continue with the immunohistochemical staining protocol.

Enzymatic epitope retrieval

The enzyme to use will be indicated on the antibody datasheet. If not, trypsin is 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 by immersing them in a container of enzyme solution. If using an automated staining system (eg Ventana), consult the manufacturer for an appropriate enzymatic retrieval protocol.

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
- Enzymatic antigen retrieval solution (for trypsin, see below)

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 solution and pre-heat to 37°C. Carefully blot excess water from around the tissue section and pipette the enzyme solution (generally 50–100 µL will suffice) onto the section. It may be necessary to spread the solution around the section with the pipette 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 min (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 min.
4. Continue with immunohistochemical staining protocol.

Immersion method

Materials and reagents:

- 37°C water bath
- Slide racks and slide rack containers
- Enzymatic antigen retrieval solution (for trypsin, see enzymatic retrieval pipetting method)

Method:

Be sure to read the manufacturer's literature for the enzyme you choose, as some enzymes require specific buffers and cofactors for activity.

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.

Use a sufficient volume of water or buffer to cover the slides.

2. Deparaffinize and rehydrate sections as above. Place slides in one water container to warm.

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.

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.

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.

4. Transfer the warmed slides into the enzyme solution for 10–20 min with intermittent gentle agitation, then remove the slides and place them in running tap water for 3 min to rinse off the enzyme.

Retrieval time should be optimized by incubating the tissue section in the enzyme solution for 10, 15, 20, 25 and 30 min before immunohistochemical staining.

5. Continue with immunohistochemical staining protocol.