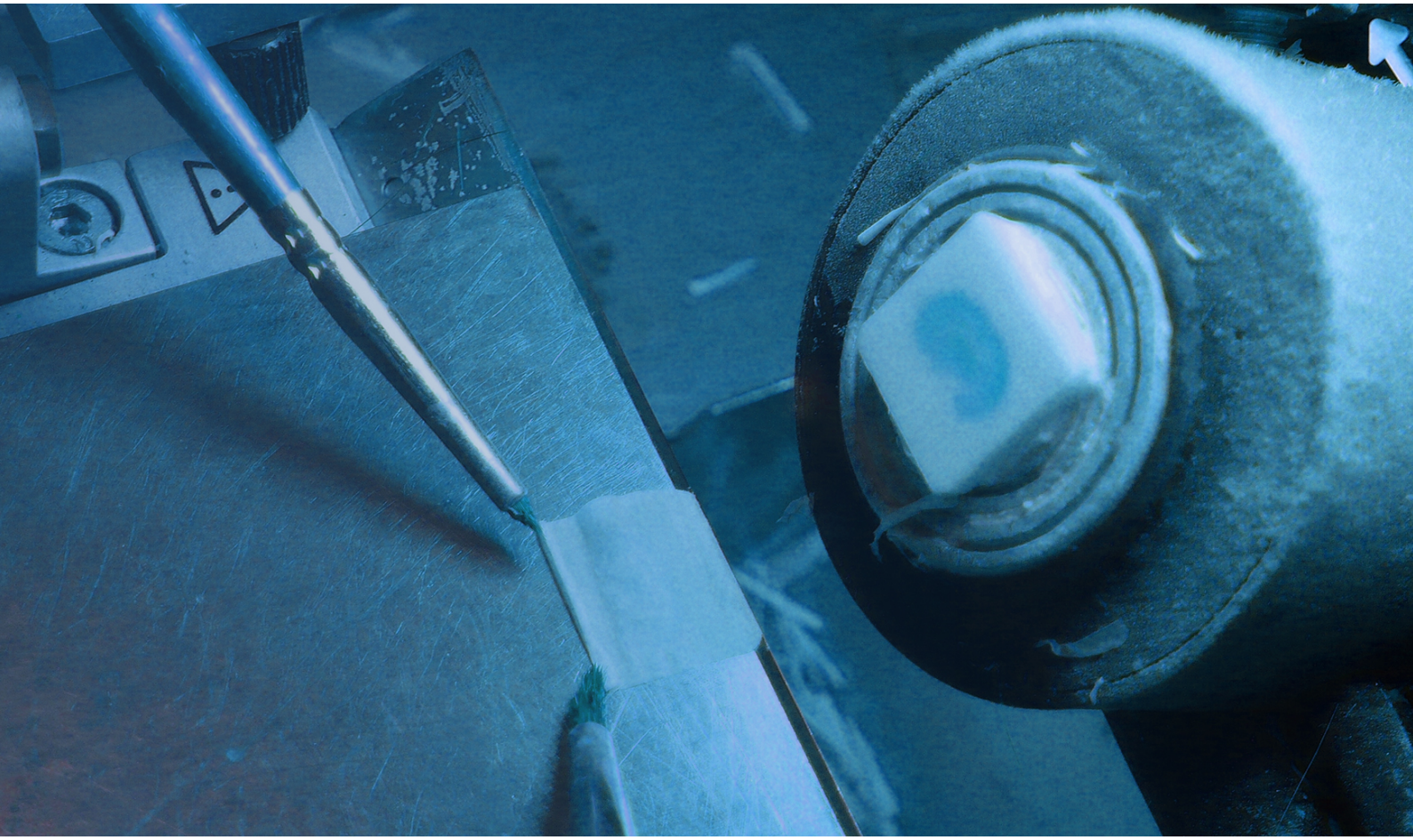
IHC-PARAFFIN PROTOCOL (IHC-P)



Creative Diagnostics

IHC-Paraffin Protocol

Creative Diagnostics provides an IHC-paraffin protocol to support your research.

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01 Introduction to IHC-Paraffin

IHC, also known as immunohistochemistry, is a technique used to show where and how many proteins are present in tissue samples. It allows the monitoring of processes in the context of intact tissue, although it is quantitatively less sensitive than immunoassays like Western blotting or ELISA. This is especially helpful for monitoring the response and progression of diseases such as cancer. In general, the knowledge gained by combining IHC and microscopy literally creates a "big picture" that can assist in making sense of data obtained by other approaches.

The target protein is recognized by antibodies used in immunohistochemical staining. Due to their high specificity, antibodies will only bind to the desired protein in the tissue section. The antibody-antigen interaction is then visualized either by chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate where the protein is located, or by fluorescence detection, in which a fluorophore is conjugated to the antibody and can be viewed by fluorescence microscopy.

IHC-P refers to the staining of tissue that has been fixed (usually in neutral buffered formalin) and paraffin-embedded prior to sectioning. The basic steps of the IHC-P approach are as follows:

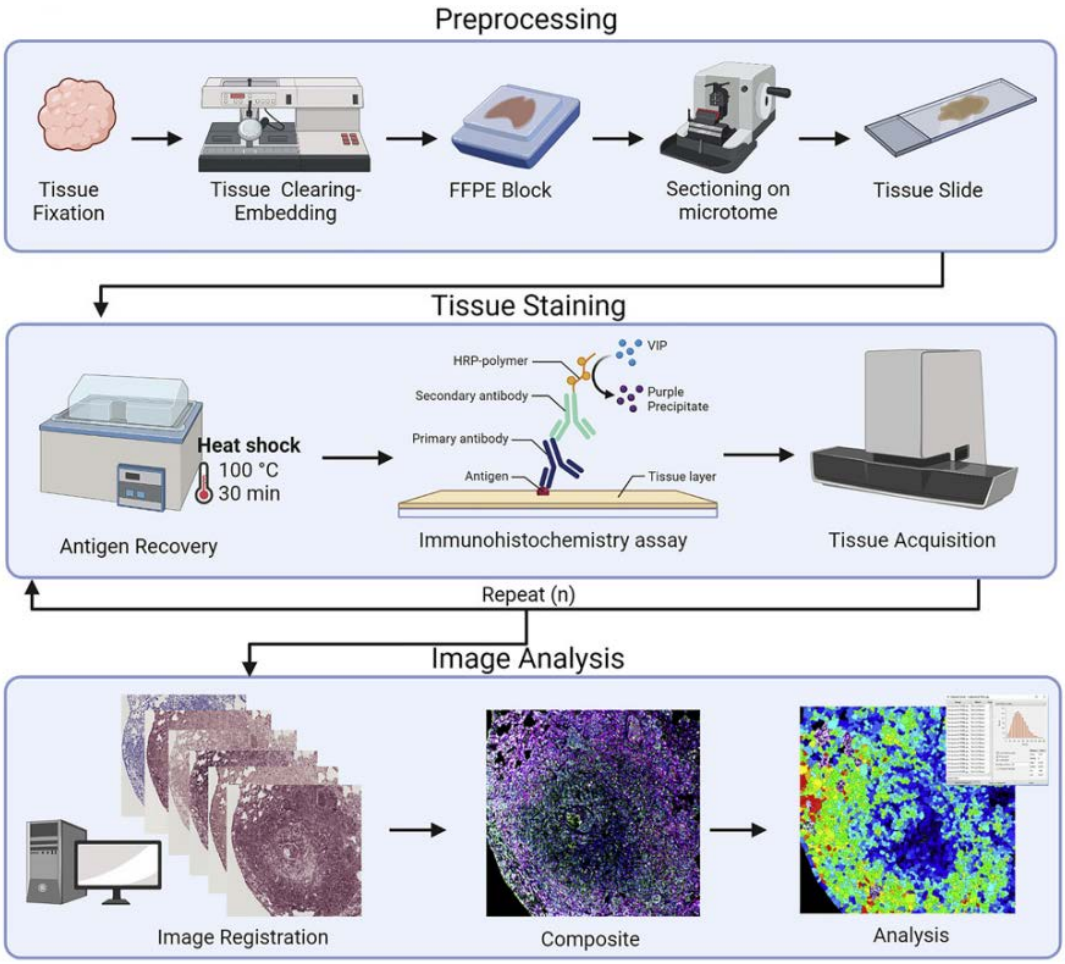

Figure 1. Immunohistochemistry for staining and analysis of paraffin-embedded tissue sections. (Maiques O, *et al.*; 2022)

1. Tissue fixation and embedding
2. Sectioning and mounting
3. Deparaffinizing and rehydration
4. Antigen retrieval
5. Immunohistochemical staining
6. Counterstaining (if required)
7. Dehydration and stabilization in mounting medium
8. Observation of the staining under the microscope

02 IHC-P Optimization of A Novel Antibody

The best staining conditions must be determined before utilizing a new antibody in IHC-P. Each antibody has an ideal dilution and each antigen has a preferred antigen retrieval technique.

1. Antigen retrieval

Test staining both with and without antigen retrieval using the strategies listed below. The Antigen Retrieval section contains comprehensive protocols for these procedures.

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 has been determined, the antibody concentration can be fine-tuned.

2. Primary Antibody Concentration

It is recommended to try 0.5 g/ml and 5 g/ml overnight at 4 °C when the antibody concentration is given. We advise trying the following initial dilutions and a 20-fold higher dilution if the antibody is not purified.

Whole antiserum: 1/50

Ascites: 1/100

Tissue culture supernatant: undiluted

3. Detection

Horseradish peroxidase (HRP) is recommended for use in visible light microscopy. The preferred chromogen and substrate for HRP is peroxide/DAB. For fluorescence microscopy, a variety of fluorochrome-conjugated antibodies are available; the choice depends on the needs of the experiment.

03 Fixation

The success of immunohistochemistry depends on proper fixation. The most common is neutral buffered formalin (NBF) 10%. Less commonly used fixatives include paraformaldehyde (PFA) and Bouin solution (formalin/picric acid).

The size of the tissue block and the type of tissue will determine the appropriate fixation duration, but fixation for the majority of applications seems to be between 18 and 24 hours. Over-fixation can hide the epitope; under-fixation can cause edge staining, where the edges of the section show high signal but the center shows no signal. Antigen retrieval can aid in removing this masking, but if the tissue has been fixed for an extended period of time (e.g., over a weekend), there may still be no signal.

After fixation, the tissue block is embedded in paraffin, cut to the desired thickness (about 5 microns is excellent for IHC), and sectioned with a microtome. Ideally, tissue sections should be mounted on slides that are positively charged or coated with APES (amino-propyl-tri-ethoxy-silane). To remove any water that may have been trapped under the section after mounting, the slides must be dried. This can be accomplished by leaving the slide at room temperature overnight. If the specimen is having difficulty adhering to the slide, the slide can be incubated at 60°C for a few hours.

04 Deparaffinization

The slides must be deparaffinized and rehydrated prior to staining. Poor section staining may result from incomplete removal of paraffin.

Materials and reagents

- Xylene
- 100% ethanol
- 95% ethanol

Method

Place the slides on a rack and wash them as follows:

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 for rinsing

Until you're ready to perform antigen retrieval, keep the slides submerged in the tap water. The slides should never be allowed to dry after this step. Drying will result in non-specific binding of antibodies and significant background staining.

05 Antigen Retrieval

Before immunohistochemistry staining can be performed on most formalin-fixed tissue, an antigen retrieval step is necessary. This is because during fixation, methylene bridges are formed that cross-link proteins, thereby obscuring antigenic regions. Enzymatic and heat-mediated antigen retrieval are the two techniques. HIER stands for heat-induced epitope retrieval.

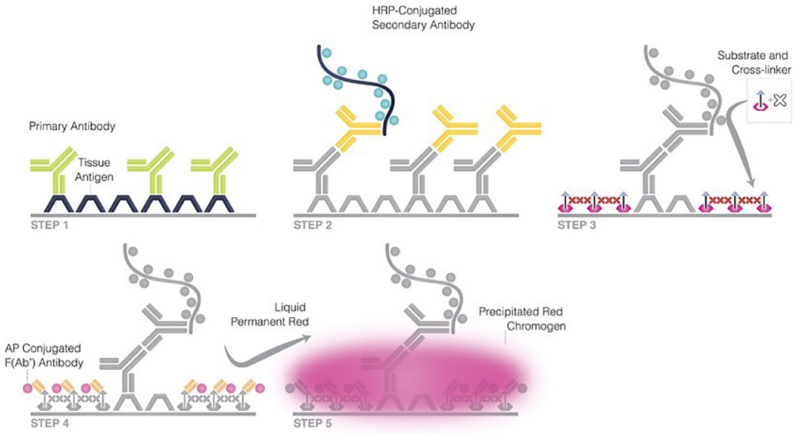

Figure 2. 1): Primary antibody binds antigen after antigen retrieval. 2): Primary antibody is recognized by a HRP-labeled secondary antibody. A pre-determined fraction of the secondary antibodies is labeled. 3): Enzyme substrate is added and deposited. 4): AP-labeled antibody binds substrate and cross linker, resulting in. 5): Red dot reaction. (Jensen K, *et al.*; 2017)

To enable the antibodies to attach, both antigen retrieval techniques work to disassemble methylene bridges and reveal the antigenic regions. Some antigens prefer heat-mediated antigen retrieval to enzymatic antigen retrieval and vice versa. Enzymatic retrieval can occasionally harm the section's morphology, so it's important to test the concentration and treatment duration. Most antigens can be recovered using a Tris/EDTA pH 9.0 buffer. pH 6.0 sodium citrate is also frequently employed.

The most popular devices for heat-induced epitope retrieval are a pressure cooker, a microwave, or a vegetable steamer. Additionally, some labs incubate slides in the retrieval solution overnight while using a water bath that is heated to 60°C. The ideal technique for each antigen must be discovered experimentally, unless the antigen retrieval method is specified on the antibody datasheet. To determine the retrieval method that provides the best staining, Creative Diagnostics recommends that several different approaches be explored.

1. Buffer solutions for heat-induced epitope retrieval

Three of the most popular HIER buffers are provided in the following solutions. The best way to choose a buffer in the absence of expert assistance from other researchers for a certain antibody is through experimentation.

- **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 4°C 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 for 10x, 50 ml for 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 4°C for longer storage.

2. Heat-induced epitope retrieval methods

a) Pressure cooker

For this procedure, the should be placed in a metal rack.

Figure 3. Heat-induced epitope retrieval by a Pressure cooker.

Materials and reagents

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

Method

1. Add the proper antigen retrieval buffer to the pressure cooker. Place the pressure cooker on the hot plate and turn up to full power. The pressure cooker's cover should only be placed on top at this time, do not squeeze the lid tightly. Deparaffinize and rehydrate the pieces as above while waiting for the pressure cooker to come to a boil.
2. After boiling, move them from the tap water to the pressure cooker. CAREFULLY USE HOT SOLUTION AND FORCESPS! Tighten the pressure cooker lid according to the manufacturer's instructions.
3. After the pressure cooker has reached its maximum (according to the manufacturer's instructions), wait 3 minutes (see note i).

Notes and tips

i. The recommended antigen retrieval time of three minutes is merely a starting point. Less than three minutes may result in inadequate antigen retrieval and poor staining. Greater than three minutes may result in over-retrieval, which could lead to nonspecific background staining and increase the possibility of sections detaching from the slides. To determine the ideal antigen retrieval time for the specific antibody being used, a control experiment should first be performed in which slides of the same tissue section are retrieved for 1, 2, 3, 4, and 5 minutes prior to immunohistochemical staining.

ii. This is to allow the slides to cool sufficiently for handling, so that the antigenic site can regenerate after exposure to such high temperatures.

b) Microwave

Domestic microwave usage is not advised. Hot and cold spots are common, resulting in uneven antigen retrieval. Due to the absence of a pressurized environment, antigen retrieval durations are typically longer, almost always resulting in section dissociation. It is considerably more acceptable to use a scientific microwave. To prevent section dissociation, most brands have pressurized tanks on board that can maintain a constant temperature of 98°C. When using this technique, a significant portion of the retrieval buffer may evaporate if the buffer boils over. Keep an eye on the slide vessel's buffer level and add more 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 holding 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. Rehydrate and deparaffinize the sections as previously described.
2. Fill the microwaveable vessel with the proper antigen retrieval buffer (see Note i).
3. Remove the slides from the tap water and place them in the microwaveable container. Put the container in the microwave. If using a home microwave, preheat on full power and run until the

solution boils. At this point, let it boil for twenty minutes. If using a scientific microwave, set the timer for 20 minutes after the temperature reaches 98°C to recover the antigens. (see Note ii).

4. Remove the vessel after 20 minutes and fill it with cold tap water for 10 minutes. Use caution with hot solutions. (see note iii).
5. Perform the immunohistochemical staining protocol.

Notes

i. If using a non-sealed jar, make sure there is enough antigen retrieval solution to cover the slides by at least a few centimeters in order to account for evaporation during the boil. During the procedure, be sure to keep an eye out for boiling over and evaporation, and don't let the slides become too dry!

ii. The recommended antigen retrieval time is only 20 minutes. Less than 20 minutes may result in inadequate antigen retrieval and poor staining. More than 20 minutes may result in over-retrieval, which could lead to nonspecific background staining and increase the possibility of sections coming off the slides. To determine the ideal antigen retrieval time for the specific antibody being used, a control experiment should first be performed in which slides of the same tissue section are retrieved for 5, 10, 15, 20, 25, and 30 minutes prior to immunohistochemical staining.

iii. This enables the antigenic site to reform after exposure to high temperature and allows the slides to cool sufficiently for handling.

c) Vegetable steamer

For heat-mediated antigen retrieval, a rice cooker or vegetable steamer is commonly used in laboratories. The process is comparable to microwaving in that it maintains the buffer's temperature at 100°C without the microwave method's intense boiling. Instead of using a steamer, this procedure can be modified to use a water bath heated to 100°C.

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

Vegetable steamer

Vessel with slide rack for approximately 400-500 ml (or 250 ml if using Tissue –Tek containers)

Antigen retrieval buffer (e.g., Tris/EDTA pH 9.0, and sodium citrate pH 6.0.)

Method

1. Rehydrate and deparaffinize the portions as before.
2. Prepare the vegetable steamer by setting it up as directed by the manufacturer.
3. Use a microwave to quickly heat the appropriate antigen retrieval buffer to boiling in a flask.
4. Insert the container holding the slide rack into the vegetable steamer.
5. Carefully add the slide rack to the container, then add the hot buffer. If it's more convenient, place the buffer in the container before putting it in the steamer.
6. Cover the steamer's lid. The buffer container should have a lid as well. The temperature of the AR solution will initially drop due to the slide rack, but will quickly rise again to 95 to 100°C.

7. After this, continue to steam the container for another 20 minutes. For the microwave approach, see Note ii.
8. Remove the vessel after 20 minutes and fill it with cold tap water for 10 minutes. Exercise caution with hot solutions. See Note iii for microwave approach.
9. Perform the protocol for immunohistochemical staining.

3. Enzymatic antigen retrieval

The antibody data sheet will indicate the enzyme options available. If not, trypsin has been demonstrated to be helpful for a variety of antigens that require retrieval after formalin/PFA fixation. There are at least two ways to apply the enzyme solution to the tissue: either by directly pipetting it onto the tissue on the slide, or by submerging a rack of tissue slides in the solution. The first approach uses less reagent, but because each slide must be handled separately, it is important to carefully monitor the incubation time of each slide to ensure that each slide is being treated equally.

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.)

Trypsin is utilized in the next procedure. Commercially accessible trypsin preparations tailored for IHC are also available, 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 ºC.
- Calcium Chloride Stock Solution (1%)

  Calcium chloride 0.1 g

  Distilled water 10 ml

  Mix well and store at 4 ºC.
- 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 ºC for one month or -20 ºC for long term storage.

Method

1. Prepare and heat the Trypsin to 37 °C. Carefully blot off the excess water from the tissue section before pipetting the enzyme solution (usually 50 to 100 ul is sufficient) onto the section. It may be necessary to use the pipet tip to disseminate the solution throughout the portion, taking care not to damage the tissue.
2. Place the slides in a humidified container and incubate at 37°C incubator. The intensity of the staining may be affected by temperature changes when the slides are placed directly on the incubator shelves. The container housing the slides should ideally be heated in the incubator.
3. Remove the slides from the incubator after 10 to 20 minutes (this will need to be tuned) and place them on a rack in a bucket of tap water. Run the faucet for three minutes to rinse.
4. Proceed with the protocol for immunohistochemical staining.

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 the temperature of the water bath to the temperature recommended for the enzyme you are using. Pour ultrapure water into two containers that can hold sliding racks. To warm the containers, add them to the water bath. (see note ii).
2. Dewax and rehydrate the sections as before. Slides can be warmed in a single water bottle (see note iii).
3. In the other container, prepare the enzymatic antigen retrieval buffer using the warm water, and then put the container back in the water bath so the solution can re-heat (see Note iv).
4. After adding the warmed slides to the enzyme solution, stir them gently every 10 to 20 minutes (see Note v). Then, remove the slides from the enzyme solution and rinse them in running water for three minutes to eliminate any remaining enzyme.
5. Proceed with the protocol for immunohistochemical staining.

Notes

i. Before selecting an enzyme, carefully read the manufacturer's literature because some enzymes require specific cofactors and buffers to function.

ii. Cover the slides with enough water or buffer to completely submerge them.

iii. Submerging cold slides in the enzyme solution will cause the solution's temperature to drop, limiting the activity of the enzyme and the ability to fully recover the antigenic site.

iv. Perform the enzymatic antigen retrieval solution as quickly as possible to avoid compromising the functionality of the enzyme. Reheat the solution before inserting the slides.

v. The suggested incubation time is ten to twenty minutes. Antigens that have been under-retrieved for less than 10 minutes may result in poor staining. More than 20 minutes may cause them to be over-retrieved, leading to non-specific background staining as well as a higher risk of section detachment or tissue morphology damage. It is advised to perform a control experiment first in which slides of the same tissue segment are incubated in the enzyme solution for 10, 15, 20, 25, and 30 minutes before being stained with an antibody to determine the ideal antigen retrieval time.

06 Immunohistochemical Staining

1. General guidelines

The following methodology is based on the assumption that the laboratory lacks an automated stainer or another capillary gap technology that enables quick application and reagent washing. If automated and semi-automatic systems are available, the steps of the protocol can be modified to work with them. Reagents can also be applied manually with a pipet.

To prevent the tissue from drying out, all incubations should be performed in a humidified space. Drying will result in non-specific binding and ultimately excessive background staining at any stage. As long as the slides are kept off the paper and can be laid flat to prevent the reagents from draining off, a shallow, plastic box with a tight top and moist tissue paper in the bottom works as an appropriate chamber! Cutting a plastic serological pipette into lengths that fit your incubation chamber is a suitable solution. Attach them in pairs to the bottom of the chamber, with the two separate tubes of each pair about 4.0 cm apart. This will give the slides a flat, raised surface to rest on, separate from the soggy tissue paper. The primary and secondary antibody dilutions are specified by testing a range or are provided on the datasheets.

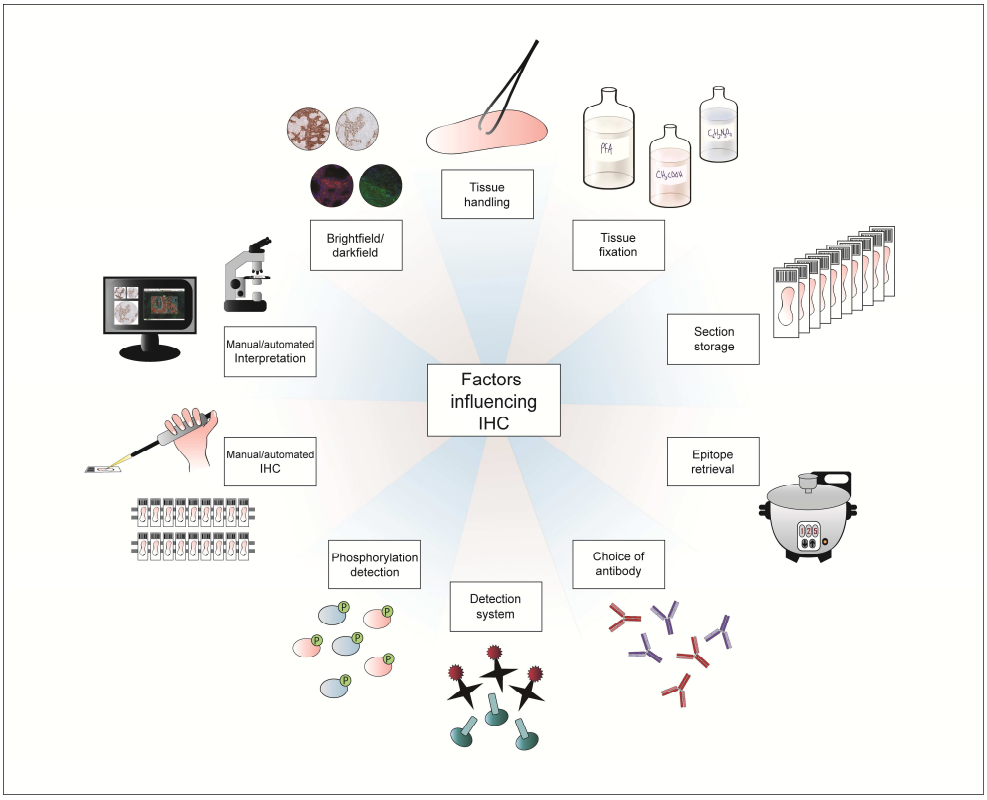

Figure 4. A schematic representation of various factors which may influence the standardization and reproducibility of the IHC process.(O'Hurley G, *et al.*; 2014)

Adjust dilutions based on results. Follow the protocol's incubation times to the letter.

The most commonly used enzymes for enzymatic procedures are horseradish peroxidase (HRP) or alkaline phosphatase (AP). These enzymes work with a variety of chromogens (see note x).

2. Protocol

Day 1

1. If utilizing an HRP conjugate for detection, endogenous peroxidase blocking can be done here, but it is recommended to wait until after the primary antibody incubation. Refer to Day 2, step 2, and Note v.
2. Gently agitate the slides while washing them twice for five minutes in TBS + 0.025% Triton X-100. Refer to 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 brief period of time (do not rinse) and then wipe tissue paper all around the sections (see Note iv).
5. Apply 1% BSA-diluted primary antibody in TBS (see Note v).
6. Overnight incubation at 4 °C (See Note iv).

Day 2

1. Rinse twice for five minutes each with TBS 0.025% Triton.
2. Incubate the slides in 0.3% H2O2 in TBS for 15 minutes if employing an HRP conjugate for detection (See Note v.).
3. For enzymatic detection (HRP or AP secondary conjugates), apply an enzyme-conjugated secondary antibody to the slide and incubate it for an hour at room temperature. The manufacturer's suggested concentration of the antibody should be diluted in TBS with 1% BSA.

For fluorescent detection:

Apply fluorophore-conjugated secondary antibody to the slide and incubate it for an hour at room temperature for fluorescence detection. The secondary antibody should be diluted to the manufacturer's suggested concentration in TBS with 1% BSA.

To avoid photobleaching, this step should be performed in complete darkness.

4. Rinse three times for five minutes each with TBS.

If fluorescence detection was used, stop here and cover the slip with mounting medium.

Continue with the next steps if you're using a chromogen to visualize the protein.

5. Develop with chromogen for 10 minutes at room temperature (see Note vi.).
6. Rinse under running water for five minutes.
7. Counterstain if necessary (see Note vii).
8. (See Note ix and x) Dehydrate, clarify, and mount.

Notes and Tips:

i. By lowering the surface tension with 0.025% Triton X-100 in TBS, reagents can easily cover the entire tissue segment. Additionally, it is thought to degrade Fc receptors, reducing non-specific binding. TBS is suggested by Creative Diagnostics as having a better background than PBS.

ii. Endogenous immunoglobulins found in the tissue may cross-react with the secondary antibody. This is minimized by pre-treating the tissue with normal serum from the species in which the secondary antibody was raised. The primary and secondary antibodies' ability to attach to Fc receptors is also eliminated when normal serum is used prior to the primary antibody administration. The inclusion of BSA reduces non-specific binding due to hydrophobic interactions.

iii. The manufacturer's guidelines or a previously diluted primary antibody should be followed. In IHC-P, most antibodies are utilized at concentrations between 0.5 and 10 g/ml. It is necessary to confirm that the primary antibody is generated on a distinct species from that used to stain the tissue. For example, an anti-mouse IgG secondary antibody would bind to all the endogenous IgG in the mouse tissue if your primary antibody was generated in a mouse and you used mouse tissue. This would result in a high background.

iv. By allowing additional time for antibodies to bind, overnight incubation enables the use of antibodies with lower titers or affinities. In addition, once the tissue reaches saturation point, no further binding is possible, regardless of the antibody's titer or affinity for its target. Overnight incubation ensures this.

v. Endogenous peroxidase activity is suppressed by H_2O_2, which reduces background staining. After rehydrating a tissue slide, place it in a DAB solution to see if endogenous peroxidases are present. A blocking step should help reduce this staining if certain regions of the section appear brown under the microscope. Some epitopes undergo peroxide modification, which reduces antibody-antigen binding. This issue is avoided by incubating sections with peroxide after the initial incubation.

   Water or TBS can be used to dilute the peroxide. When peroxide is diluted with methanol, it tends to reduce the tissue damage caused by the reaction in aqueous solutions, which is advantageous for blood smears and other peroxidase-rich tissues. However, for other tissues, dilution in TBS or water is recommended. After incubation with methanol and peroxide, the binding of various antibody-antigen pairings is reduced, especially cell surface proteins. Peroxidase quenching should not be used with AP or fluorescence detection because it only affects HRP conjugates.

vi. Prepare the colored enzyme product using the proper chromogen. The decision is based on your preference for the color of the final result, the enzyme label you're using, and whether you're using aqueous or organic mounting media (see Note xii for more information).

vii. Hematoxylin (blue), nuclear fast red, or methyl green are some examples of counterstains that are frequently utilized. DAPI (blue) or propidium iodide/PI (red) can be utilized as a counterstain when using fluorescence detection.

viii. Remember that DAB is thought to be a possible carcinogen. Put on the proper protection gear. Dispose of it in accordance with laboratory instructions after deactivation with chloros in a sealed container overnight (the addition of chloros causes poisonous fumes). Levamisole (Sigma L9756), 0.24 mg/ml, should be added to the chromogen solution when using AP. Levamisole lowers background staining by inhibiting endogenous phosphatase activity.

ix. Remember that all aqueous chromogens, including AEC, Fast Red, INT, and others, are soluble in alcohol when utilizing them. Utilize an appropriate aqueous mounting medium. Avoid dehydration and clearing!

x. Dehydrate and clear any organic chromogen produced sections, such as DAB, New Fuchsin, Vega Red, NBT, or TNBT by following the rehydration procedure in reverse order. Mount the sections in an appropriate organic mounting medium. The refractive index of sections mounted in organic mounting media is higher than that of sections mounted in aqueous mounting media. This means that the image seen through the microscope is cleaner and sharper when organic mounting media is used.

References

1. Jensen K, et al.; A novel quantitative immunohistochemistry method for precise protein measurements directly in formalin-fixed, paraffin-embedded specimens: analytical performance measuring HER2. Mod Pathol. 2017, 30(2):180-193.

2. Maiques O, et al.; Multiplex chromogenic immunohistochemistry to stain and analyze paraffin tissue sections from the mouse or human. STAR Protoc. 2022, 3(4):101879.

3. O'Hurley G, et al.; Garbage in, garbage out: a critical evaluation of strategies used for validation of immunohistochemical biomarkers. Mol Oncol. 2014, 8(4):783-98.

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