



# The Michigan MIKRO-GRAF

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## **IHC PRETREATMENTS** *What are they and why do we do them?*

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HIER, AR, EIER, PIER... what do all of these abbreviations mean? In the world of immunohistochemistry (IHC), these are all abbreviations for different ways to pretreat tissue sections before staining begins. Pretreatment is often necessary due to the effects of fixation on tissue sections, and its purpose is to try to expose and unmask the binding sites where IHC staining will occur. This article will explain some basics of fixation, IHC staining, and some different methods of pretreatment that are used in today's labs and why they are used.



**Tech Points**

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## **LABORATORY STAFF SHORTAGE** *How are we promoting our profession?*

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MSH President

At some point in your career, you have been or will be asked the question: "What do you do for a living?"... At this point there is usually a pause where you formulate a response. If you respond "I'm a histotech" or "I'm a histotechnologist", most likely you were asked "What exactly is that?" The need for histotechs in the country and in Michigan is growing. Increased retirement and an aging population have attributed to the estimated increase of laboratory professional job openings. The problem is few people know the profession is available.

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(IHC Pretreatment, cont. from page 1)

Neutral buffered formalin is the most common fixative in today's histology laboratories. It is a universal fixative that preserves tissue structure and cell

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morphology and is easy to purchase, store, and dispose of.

Unfortunately, formalin works to stabilize the tissues by

forming crosslinks between proteins within that tissue. The crosslinks that are formed are strong bonds that can cause the configuration of the tissue proteins to fold and twist upon each other, thus altering the natural molecular configuration and masking potential antibody binding sites. For routine hematoxylin and eosin (H & E) staining and most special stains this abnormal configuration is not an issue so pretreatment is not necessary, however for some immunohistochemical reactions these effects of fixation need to be reversed.

The basis of IHC staining is an antibody – antigen interaction. The antigen is present within the tissue section. An antibody that has been produced outside of the body against that antigen is added to the tissue section and the two will bind. Then a detection system is added, which is a series of reagents that ultimately bind to each other and yield a colored end product so we can visualize the antibody – antigen interaction.

The site of an antigen where the antibody will bind is called an epitope, and is a unique specific combination of amino acids present within an antigen. When the formalin ‘fixes’ the tissues and creates the crosslinks that stabilize and preserve the tissues, the epitopes are sometimes masked or hidden due to the twisting of the molecule on itself. “Pretreatment” is a generic term for a method of treating the tissue where the fixation crosslinks are broken and the epitope is exposed and becomes available for binding. Pretreatment is generally done by the addition of heat or by digestive enzymes that break these crosslinks.

Primary antibodies prefer specific pretreatment procedures, however at times performing multiple pretreatment procedures will still yield acceptable IHC staining. The optimal suggested pretreatment should be determined by the primary antibody manufacturer and listed on the data sheet, although at times your lab may find a different pretreatment method to work better. It is often recommended to try a battery of pretreatment methods for each primary antibody to find your optimal procedure in your lab.

In any of these procedures that require pretreatment, it is recommended that positively charged slides be used. The addition of heat or digestive enzymes can be rigorous on the tissue and tissue fall-off is often experienced. Also extended slide drying times can be helpful to retain tissues during pretreatment.

### Heat Induced Methods

Heat Induced Epitope Retrieval (HIER, at times called Antigen Retrieval or AR) is the application of heat using different heating sources while immersing the slides in a buffered solution to break the crosslinks and ready the epitope for binding to the antibody. Tissue sections are deparaffinized and rehydrated to water. Then a buffer is chosen that is preferred to the antigen that is being detected. These buffers are made with different chemicals and are regulated to maintain different pH's. Common buffer solutions at usual manufactured pH's for HIER are:

- Citrate buffer at pH 6.0
- EDTA buffer at pH 8.0
- Tris buffer at pH 10.0

There are several other buffer solutions that can be used and that are preferred by specific antibodies however these are the most common in the clinical IHC lab.

Common heating elements used for HIER are:

- Vegetable steamer
- Pressure cooker
- Microwave
- Waterbath
- Rice cooker

Slides are heated to between 90°C and 120°C, depending on the heating element used. A waterbath will not heat solutions to as high of a temperature as a pressure cooker will, due to the restraints of the heating method and equipment. Also different methods will keep the tissues at these elevated temperatures for varying amounts of time. For example, using the pressure cooker for HIER will keep the slides at a high temperature (and under pressure) for a short amount of time (as short as 1 minute). This is a harsh pretreatment, compared to using a vegetable steamer that keeps slides at a lower temperature for a longer amount of time (i.e. 96°C for 25 minutes), which is a gentler pretreatment for the tissue sections.

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(IHC Pretreatment, cont. from page 3)

In addition to using different buffers for different antibodies, different heating methods are sometimes also preferred by specific antibodies. The primary antibody data sheet that is provided by each manufacturer, with the antibody, should give the recommended beginning guideline for which pretreatment method to use.

### Digestive Enzyme Methods

Some antibodies prefer a digestive enzyme pretreatment instead of the high heat methods (EIER – Enzyme Induced Epitope Retrieval). After deparaffinization and rehydration, a digestive enzyme is added to the tissue for generally 5 to 20 minutes, at times with the addition of gentle heat (37°C). Examples of digestive enzymes are:

- Pepsin
- Trypsin
- Protease
- Pronase

These enzymes are also called proteolytic enzymes (PIER – Proteolytic Induced Epitope Retrieval), as they break down proteins into smaller pieces thus breaking the crosslinks and exposing the epitopes. Each enzyme will break the fixation crosslinks at different sites.

### Pretreatment Battery

Since specific antibodies prefer different pretreatments, and fixation effects can vary due to times or reagents used, a recommendation is to have a set battery of pretreatments to use in your lab to determine the optimal pretreatment conditions for each antibody when they are used in your hands.

A recommended battery of pretreatment methods to try might include:

1. No pretreatment
2. Citrate buffer, pH 6.0 in vegetable steamer, 25 minutes at 96°C
3. EDTA buffer, pH 8.0 in vegetable steamer, 25 minutes at 96°C
4. High pH buffer, ie. Tris buffer, pH 10.0 in vegetable steamer, 25 minutes at 96°C
5. Pepsin digestive enzyme, 37°C for 15 minutes
6. Protease digestive enzyme, room temperature for 10 minutes

If a different method of heating is used routinely in your lab (like a pressure cooker) then use the buffers above (citrate, EDTA, and Tris at the recommended pH's) in

the method that you are used to at the most standardized temperature and time. For example if you generally use a pressure cooker for 2 minutes at 100°C for most of your antibody pretreatments, use this with the buffers listed above.

Present the 6 slides to your pathologist(s) and leave it to their discretion as to what pretreatment method is the best. Do these tests on a piece of control tissue that is known to be positive (usually on the weakly-positive side, to ensure that you can find those weaker-expressing cells), and that has been routinely fixed and processed.

An example of specific antibodies preferring different pretreatments is seen with CD3 (clone PS1). If using the battery recommended above, the results one might see include:

1. No pretreatment – no staining
2. Citrate buffer – light staining
3. EDTA buffer – dark specific staining
4. Tris buffer – overly dark staining, tissue fall-off
5. Pepsin- no staining
6. Protease – faint staining

These slides could be evaluated and through your experiments, it could be determined that CD3 (PS1) would be best with an EDTA buffer pretreatment in your lab.

Pretreatment is an integral step in IHC staining and its importance should not be overlooked or simplified. If you are having trouble with a particular antibody not working, the pretreatment method being used could be a major part of your troubleshooting procedure. Remember that different antibodies prefer different pretreatments, and in your hands it could be different than what a manufacturer recommends. There are many different ways to pretreat tissues prior to IHC, so be sure to realize this when working up an antibody.

### References

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