

## **Preparation of frozen sections for IHC**

Frozen sections for melanoma testing should adhere to the same standards that apply to any Mohs section (i.e. 4-5 microns thick and a complete section with all of the epidermal edge). To obtain the necessary testing material a slide needs to be cut from each fresh tissue section to be tested. Considerable extra time and effort may be needed to cut the frozen section slides for a melanoma case if the Mohs layer is of a larger size and involves significant adipose tissue and needs to be divided into many sections for processing. Using as an example a 20 mm diameter Mohs layer cut into four sections for fresh frozen processing, four slides will be cut for the standard hematoxylin and eosin staining plus the four additional slides needed for IHC testing. Control slides should be cut at this time as well.

Testing fresh frozen tissue requires the use of fresh frozen tissue controls. There needs to be a positive and negative control slide for each antibody being used and a negative methods control slide for each staining run. The positive control slide and the negative methods slide are cut from a saved frozen tissue block that has previously shown to give a positive result for the antibody being used. The negative methods slide receives no primary antibody during the staining run. Negative control tissue for melanoma would ideally be cut from a saved frozen tissue block that has no melanocytes. An additional seven slides then need to be cut for a single IHC run with one antibody so that eleven frozen section slides total would need to be cut. (It might also be a practical concern that cutting deeper into the frozen block to obtain the extra slides needed for IHC may yield false positive results.)

The slides for IHC then need to be fixed, air dried, or a combination of both before staining. Acetone is generally used as the fixative for fresh frozen tissue because acetone is non-additive in that it evaporates rapidly from the tissue and it dehydrates and fixes the tissue quickly. A coplin jar of acetone can be placed in the cryostat for convenience. As most of the reagents used in the IHC process such as the Tris buffer solution have a short shelf life, they should be prepared immediately before a staining procedure, hopefully a few hours before starting the staining procedure.

## **Validation**

For smaller Mohs laboratories, validation requires a lot of time-consuming effort, but it is a necessary and very important preceding component of the IHC procedure. Anything that affects the outcome of the IHC process such as the controls and their storage condition, fixation, the antibodies and the reagents all need to go through the validation process to assure their efficacy. The temperature and humidity of the lab can also be a factor in the consistency of the staining results, so these aspects need to be monitored and controlled. Written logs must be kept of all the validations to ensure reproducibility and satisfy CLIA standards.

Each batch of a particular antibody needs to be tested to determine whether the optimal incubation time is the same as the last batch of that same antibody. If concentrated antibodies rather than prediluted staining kits are being used, their proper dilutions need to be determined and validated. The main goal of the

antibody validation process is to optimize the specific staining while determining a reasonable incubation time and reducing background staining. The difference between the intensity of the antigen being stained and unwanted background staining is called the “signal to noise” (SN) ratio. The sensitivity or attractiveness of an antibody to a specific antigen is called an “affinity” to that antigen. The minimal amount of time required for an antibody at optimal dilution, or the “functional affinity”, to saturate a specific antigen is called the “equilibrium point”. At this equilibrium point, not only are positive results easier to visualize, the antibody-antigen reaction is also less likely to disassociate during the subsequent washing cycles of the staining procedure.

Monoclonal antibodies have a lower antigen affinity but are more antigen-specific and more expensive to use than polyclonal antibodies. Prediluted antibodies are designed to work with the kit reagents they were packaged with, and substitutions for these reagents can lead to undesirable and unpredictable results. As such, the type of antibody used can make a difference in the staining protocol that is chosen.

### **Selection of staining method**

There are peroxidase-labeled (horse-radish peroxidase or HRP, aka IPOX) antibodies and there are alkaline phosphatase (AP) labeled antibodies. The enzyme labeling makes possible the colored chromogen staining of the antigen of interest by means of a chemical reaction. HRP antibodies were the original antibodies used for IHC and are the more familiar and commonly used. Peroxidase-anti-peroxidase (PAP) and peroxidase-peroxidase-anti-peroxidase (PPAP) staining methods employed using single or multiple HRP antibodies. Although stacking these antibodies into a complex of antibodies provides better staining of the antigen of interest, this often increases endogenous background staining which can complicate accurate interpretation of the slides.

By combining an enzyme labeled antibody with biotin you create more binding sites for chromogenic attachment as compared to the standard peroxidase method. This avidin/biotin complex (ABC) staining method increases the SN ratio by four times. Combining an enzyme labeled secondary antibody with a biotin labeled primary antibody for what is called the labeled streptavidin/biotin (LSAB) method, increases the SN ratio even more by providing greater antibody sensitivity and signal amplification.

One challenge with the IPOX/HRP and ABC/LSAB staining methods is the presence of endogenous peroxidase and biotin in the tissue which are at even higher levels in fresh frozen tissue than in paraffin embedded tissue. Besides often being ruinous to the tissue, blocking or quenching of peroxidase and biotin adds more time and complexity to the IHC procedure. Thus, the hydrogen peroxide concentrations for pre stain blocking are kept to a minimum in order to prevent the destruction of the tissue samples.

Alkaline phosphatase labeled antibodies are derived from calf intestines. Levamisole is used to quench endogenous AP in tissue where AP might be present but it is not needed for skin tissue sections. The levamisole blocking step does not destroy skin like hydrogen peroxide does.

Using the polymer string staining method with an AP labeled antibody provides a means of avoiding both endogenous peroxidase and biotin, which eliminates the need for blocking. With this staining method, a dextran filament contains secondary antibodies to any primary antibody of rabbit or mouse origin and provides a string of chromogenic attachments analogous to a string of lights. SN ratios are as good as or

better than those achieved with the LSAB method. Although there is no need to block or quench the tissue, tris buffered saline (TBS) a combination of tris (hydroxymethyl aminomethane), sodium chloride and sodium azide) should be used to rinse slides during the staining process instead of phosphate buffered saline (PBS) in order to avoid background staining. PBS is usually used as the rinse solution that is used when using IPOX/HRP antibodies for immunostaining.

### **Melanoma antibodies**

This discussion is limited to immunostaining for melanoma, but still there is a wide choice of antibodies to choose from. (See table.) Microphthalmia transcription factor (MiTF) is perhaps the most recent of the melanoma detecting antibodies being used. MiTF regulates the development and survival of melanocytes and is also involved in transcription of pigmentation enzyme genes such as tyrosinase. The MiTF antibody recognizes a nuclear protein which is expressed in the majority of primary and metastatic epithelioid malignant melanomas as well as in normal melanocytes, benign nevi and dysplastic nevi. No matter which antibody is used, the protocols used for validation and staining should be the same.

### **Cost considerations with IHC**

There are three classes of antibodies specified by Food and Drug Administration (FDA) regulations: analyte specific reagent (ASR), in vitro diagnostic (IVD), and research use only (RUO) with the RUO antibodies being the least expensive. The class of antibody being used as well as other important descriptive information is listed on the antibody datasheet supplied by the antibody manufacturer with each antibody. A copy of the datasheet for each batch of antibody purchased must be kept for at least two years as per CLIA standards.

Antibody staining is a more expensive endeavor compared to conventional staining. The common stains used in a Mohs lab such as hematoxylin, eosin, and toluidine blue are certified for standard consistency by the International Biological Stain Commission. There is no such commission regulating the available antibodies so there is no standard of consistency. Antibodies can therefore vary from batch to batch and from wholesaler to wholesaler even when using those of monoclonal type. To test and standardize antibodies would put their price well beyond any practical utilization.

### **Chromogen color**

Chromogen substrate solutions deposit a visible color marker at the antigen of interest. With HRP substrate solutions, hydrogen peroxide is combined with a chromogen. The chemical reaction between the HRP bound to the antibody and the hydrogen peroxide yields water and the colored marker. AP substrate solutions require a naphthol phosphate bound chromogen which reacts with the AP linked antibody to release the colored marker. Diaminobenzidine (DAB) is the most commonly used chromogen with HRP antibodies while Permanent Red or New Fuchsin are the chromogens used most often with AP antibodies.

It might be unnecessary to use the (DAB) chromogen for melanoma studies. Although DAB substrate solution is easy to make and DAB is a permanent chromogen, meaning it won't be removed by exposure to a solvent, it is a carcinogen that requires special disposal protocols. It is also brown in color just like melanin. Alternative chromogen colors are available such as red, yellow, and blue or green, some of

which are also resistant to solvents like DAB so that the IHC slides can be cover slipped and stored as permanent slides.

### **Step by Step**

Going back to the initial example of a 20 mm diameter Mohs layer that has been sectioned into four pieces, the edges inked and the map drawn, the first step is to cut the fresh frozen tissue slides. While cutting the H&E slide for each section an additional slide will be cut for immunostaining. The fresh frozen control tissues are then retrieved from the storage freezer. Two slides will be cut from the positive for melanoma control tissue, and one slide will be cut from the negative for melanoma control material. The control and immuno study slides will be labeled and then given a quick dip in the acetone fixative before being placed in the staining tray. The H&E slides will be processed as per usual for diagnosis.

An IVD class, murine monoclonal, MiTF, AP-labeled antibody is being used in this example. Since no enzyme blocking is necessary the slides will first be washed with Tris solution to freshen the tissue. Tip each slide to the side to drain off any excess Tris from the slides. Add the antibody to the test slides and the positive and negative control slides. Do not put any antibody solution on the negative methods slide.

After the determined incubation time has passed, the slides are carefully and gently rinsed with Tris solution with any excess Tris being tipped off to the side. Now an anti-rabbit polymer string secondary antibody is applied to every slide, even the negative methods slide. This secondary antibody will bind to the primary antibody and will provide multiple chromogen attachment sites.

The secondary antibody also needs to be incubated for a predetermined time period before being rinsed off with Tris solution. When the excess solution has been tipped off, New Fuchsin chromogen which has been properly mixed per manufacturer's instructions into solution with levamisole is applied to every slide. After a minute or two the slides can be gently rinsed in distilled water and then counterstained with H&E (optional), dehydrated with ethanol, cleared and cover slipped or just dehydrated, cleared and cover slipped without counterstaining for microscopic reading and diagnosis.

### **Conclusion**

Most pathology labs have automated the process of immuno staining. As with conventional H&E staining the meticulous nature of IHC staining lends itself to robotic/automated staining in order to achieve consistent results, improve efficiency, and reduce cost of operation. Automation has a huge initial cost of start-up though, which makes automated staining impractical for most Mohs labs that want to do their own testing. Technical advances in the field of immunohistochemistry have made doing immuno staining in the Mohs lab more practical. While the validation process is tedious, the actual staining protocol is simple and straight forward. With careful practice and experimentation it can be a useful tool in the Mohs frozen section lab.