

***TECHNICAL IMMUNOHISTOCHEMISTRY:
Achieving Reliability and Reproducibility of
Immunostains.***

Rodney T. Miller, M.D.

Director of Immunohistochemistry

ProPath Laboratory, Inc.

8267 Elmbrook, Suite 100, Dallas, Texas 75247-4009

Phone (214) 237-1631 FAX (214) 237-1770

www.propathlab.com E mail: rmiller@propathlab.com

Society for Applied Immunohistochemistry

2001 Annual Meeting

New York Cornell-Queens Hospital Medical Center, Flushing, NY

Saturday, September 8, 2001

8:30-10:00 am and 1:30-3:00 pm

INTRODUCTION

It is difficult to think of a technique that has had a greater impact on many areas of pathology than immunohistochemistry (IHC), particularly from the standpoint of diagnostic pathology. Certainly, other relatively “new” techniques such as flow cytometry and molecular diagnostics have made tremendous contributions, but in the grand scheme of things they simply cannot address the tremendous range of diagnostic problems that occur in routine surgical pathology, hematopathology, and cytopathology to the same extent as IHC can at the current time. I suspect that the emerging importance of molecular methods may at some point in the future supplant the prominent position enjoyed by IHC, although I believe the role of IHC will continue to be of prime importance, at least during my lifetime.

In order for IHC to be used effectively, it is critical that the stains be reproducible and reliable, an often elusive goal. Although in theory the technique is very simple, in practice it has been difficult for many laboratories to achieve consistent and reliable results. Indeed, there are many factors that can contribute to the quality of the final result, and in order to optimize the results, it is important to become thoroughly familiar with the technical aspects of the staining procedure, as well as understand the importance of continuous, ongoing quality control. It is my hope that this presentation will fulfill the goal of providing the attendees with an in depth background in the technical aspects and quality control of IHC, so that he or she will be able to apply some of the information gained during this course to improve results within their own labs, both from the standpoint of quality and economy.

This presentation is directed toward those who have a working knowledge of IHC, and will mainly address paraffin section-based IHC, since that is by far the most commonly used method for diagnostic purposes, although suggestions for handling cryostat sections, bone marrow smears, and cytologic smears will also be reviewed.

Since disclaimers have recently become quite popular, I will add my own at this point, and say that **all IHC laboratories are different**, and what works best for me may not be best for every laboratory. I encourage anyone to share their experiences and ideas for improvements in technique with others, since I have benefited greatly from other people's willingness to share their ideas with me.

KEY ELEMENTS OF A SUCCESSFUL DIAGNOSTIC IHC LABORATORY

1. Pathologist Director:

One key element in a successful diagnostic IHC laboratory is a knowledgeable and committed pathologist Director. It is important for the Director to have bench-level experience and in depth technical knowledge, since this is indispensable in troubleshooting problems as they arise. It is also important that the Director realize that a certain time commitment is necessary to maintain and nurture an IHC laboratory, and if the pathologist is unable or unwilling to devote the time to this area, immunostains are best performed elsewhere. I have heard a number of pathologists complain that their technologists can never get immunostains to work, and readily shift the blame for poor results onto the shoulders of the technologists. However, this is an inappropriate attitude on the part of the pathologist, since the ultimate responsibility for the quality of the stains should rest with the pathologist. A successful pathologist Director must also be committed to remaining current with the ever-expanding IHC literature, since awareness of the specificities of the antibodies and expected patterns of reactivity in tissue is critical to employ this technique effectively and avoid diagnostic or interpretive errors.

2. Technologists:

Obviously, technologists are also critical to a successful IHC laboratory. If the technologists in the laboratory are also responsible for performing the tissue microtomy, it is important that they are highly skilled, particularly in cutting numerous sections from minuscule pieces of tissue within paraffin blocks. Before technologists begin performing immunostains, it is important for them to be instructed in the theory of the technique, so that they have an understanding of the method, rather than adopting a "cookbook" approach, which will seriously hamper their troubleshooting skills. It is also important for the Director to maintain free and open lines of communication with the technologists working in the laboratory.

3. Reliable Reagents and Methods:

There are many sources of reliable high-quality reagents, and a variety of different methods are available for performing immunostains. Many manufacturers market "universal kits" that contain link antibodies and detection reagents, and may also contain blocking reagents or developing reagents. Many of these kits perform satisfactorily, although it is certainly not difficult to purchase reagents separately and develop your own method, a more economical option in many cases. I am partial to detection systems with a peroxidase marker enzyme and diaminobenzidine (DAB) chromogen, since these have been the most economical, reliable, and reproducible reagents in my experience. There are also numerous sources of primary antibodies, which are of variable quality, although many high-quality reagents are now easily obtained. One extremely important point to realize is that **you must not believe the manufacturer's claims for their antibodies before validating them in your own laboratory.** Additionally, you must also realize that **each laboratory must determine their own optimal titers, and that there is no such thing as a "predilute ready to use antibody".** (More about this later).

4. Quality control

Lax quality control is the downfall of many laboratories. Quality control should not be a spurious process performed in fits and starts (particularly immediately before an inspection), but rather should be a continuous and ongoing process that should document the sensitivity and specificity of every stain that is performed. In my experience, the extensive use of multitumor control block preparations is the key to efficient and effective quality control, and this will be discussed in greater detail later.

PREANALYTIC (PRE-STAIN) VARIABLES

Cutting the Gross Specimen

Quality in IHC starts at the grossing table, and it is important that sections for processing be cut thin (ideally 2 mm in thickness) and not crammed into processing cassettes. If good H&E sections cannot be obtained from processed material, the immunostains will also be suboptimal, and there is much truth to the saying "**GARBAGE IN-GARBAGE OUT**".

Effects of decalcification

A number of authors have evaluated the effects of decalcification on immunostains (1-5), and reported effects have varied. Personally, I have seen distinct damaging effects of decalcification on a number of antigens (particularly CD43, Ki-67, ER, and PR), although many other antigens do not appear to be adversely affected to a significant degree. Strong acids should be avoided if possible, and undoubtedly there are varying effects depending on which particular decalcifying agent is employed. An additional problem that I have observed in some cases is artifactual nuclear staining (sometimes quite strong), that seems to occur in only certain cases (from certain clients) when subjected to certain types of antigen retrieval (particularly Tris buffer). In some of these cases, performing the antigen retrieval in dilute urea has allowed us to compensate for this problem, although when studying bone marrow core biopsies for nuclear antigens such as TDT or cyclin D-1, this can be a headache. Because of these adverse effects of decalcification, I always prefer to immunostain clot sections from bone marrow biopsies (since these have not been decalcified) if they have adequate diagnostic material.

Formalin fixation

It is well known that extended fixation times in formalin are damaging to many antigens, secondary to the induction of molecular cross-links in proteins. This can change the native three-dimensional protein conformation, thereby altering the normal three-dimensional structure of the epitope (the specific area of the protein where the antibody binds), making it more difficult for the antibody to bind to its target. The magnitude of this adverse effect varies with the length of fixation, with increasing fixation times causing increasing amounts of antigen damage, also referred to as "antigen masking" (6). However, if tissue is not overfixed in formalin (which I would consider to be anything over 24 hours), formalin is actually an excellent fixative for immunohistochemistry, and in many instances the deleterious effects of formalin fixation can be easily overcome by employing appropriate epitope retrieval techniques.

Protease digestion

Protease digestion (6-9) was the first method used to counteract the antigen masking effects of formalin fixation, and a wide variety of proteases have been employed, including trypsin, DNase, proteinase K, pepsin, pronase, ficin, and others. Presumably the beneficial effects of protease treatment are related to cleavage of the molecular cross-links by the proteolytic enzyme, allowing the epitope to return to its normal conformation, enabling more effective binding by the antibody. Ficin is one of my personal favorites, and is still used in my laboratory on a daily basis for a small number of antigens. However, since the advent of heat induced epitope retrieval (HIER) techniques, proteases play a much smaller role in most IHC laboratories.

Heat Induced Epitope Retrieval (HIER), a.k.a. Antigen Retrieval

Heat induced epitope retrieval (HIER) is an alternate term for a technique originally described as "antigen retrieval", which involves placing the slides in heated solutions of various composition before application of the primary antibody. This technique was developed by Dr. Shan-Rong Shi and colleagues (10) and has literally revolutionized immunohistochemistry, since it has greatly expanded the range of antigens that are detectable in paraffin section material (10-22). Initially described using microwave as a source of heat, this technique has been also found to be effective when employing other heat sources (23-29), including hot water baths, vegetable steamers, pressure cookers, and autoclaves. Presumably HIER break the molecular cross-links induced by formalin, although the mechanism of action is not yet completely understood. There is

some evidence that precipitation of tissue bound calcium (or other divalent cations) may be important (30-31). Calcium-chelating agents (such as EDTA) can be effective epitope retrieval solutions, and some investigators have found that the beneficial effects of HIER can be reduced by adding divalent metal ions (calcium chloride or magnesium chloride) to the incubation buffer. Formalin fixation generates large numbers of hydroxymethyl groups that link to protein groups, making it possible for strong bonds to form between proteins and calcium ions. The resulting "cage-like" complex may be involved in the antigen masking effects of formalin fixation.

Interestingly, alcohol-fixed tissues (which generally are not considered to have extensive cross linking of proteins) may also benefit from epitope retrieval (14, 19). Alcohol is considered a dehydrating fixative, and is thought to exert its fixative effects by the removal of water molecules that are non-covalently bound to side chains in amino acids. Conceivably, removal of these bound water molecules has the potential to alter the native three-dimensional configuration of the protein and hence the epitope. It has been hypothesized that HIER restores bound water to the protein structures, returning the epitope to a more native state.

Drs. Shi, Taylor, and colleagues have investigated many aspects of HIER (20-22, 32), and they have found that the degree of epitope recovery is a function of a number of factors. Time of heating and temperature are involved, and equivalent degrees of epitope retrieval can be obtained by employing shorter heating times with higher temperatures, and vice versa (within certain limits of course). In addition, the pH of the HIER solution used can also affect the final results. Unfortunately, there is no single HIER solution that is best for all antigens, nor is there a single HIER heating method that is optimal for every single antigen. There is a fairly wide variety of HIER solutions that may be employed, including 0.1M citrate buffer (pH 6), 0.1M EDTA (pH 8), 0.5M Tris base buffer (pH 10), 0.05M glycine-HCl buffer, 1% periodic acid, various concentrations of urea, lead thiocyanate solutions, and others. Incubation buffer (or even distilled water) may also work well for some antigens, and some laboratories have had good results using dilute solutions of liquid hand soap (Softsoap) (Marcia Price, personal communication). It is advisable for each laboratory to evaluate a number of different epitope retrieval solutions, and choose those that work best for the particular antigens of interest.

Parenthetically, I should mention that the starting temperature of the HIER solutions used should be kept consistent. If the HIER solution is at refrigerator temperature one-day and at room temperature another day, there may be variable results obtained, since the effectiveness of retrieval varies with the temperature of the solution, as noted above. Additionally, we have observed that the pH of the EDTA antigen retrieval solution that we use (normally pH 8.0) tends to change (decrease) over time, so we have found it necessary to monitor the pH of this reagent every two days in order to ensure consistent results.

HIER with Microwave Ovens

Microwave ovens are very commonly used for HIER. There are several points that are worthy of mentioning, however. From personal experience, I now realize that every microwave oven has its own personality. In other words, don't expect to obtain identical results using two different microwave ovens, even if they share the same wattage. Each laboratory using a microwave oven must determine its own optimal microwave incubation times. Since the effectiveness of heating of microwave ovens varies with the amount of material in the oven, I believe it is important to always keep the same number of incubation jars filled with fluid (distilled water works fine) in the microwave oven at all times, even if only a small number of stains are being processed. This will aid in maintaining consistency of heating between runs. Since microwave ovens are also

notorious for having hot and cold spots, carousel microwave ovens are recommended to help minimize this problem, although these do not completely eliminate it.

HIER with a Pressure Cooker

A number of authors have reported the use of pressure cookers for HIER (28-29), and pressure cooking has a number of theoretical advantages over the use of microwave ovens. Unlike microwave ovens, pressure cookers do not have hot and cold spots, so heating is more evenly distributed and reproducible. During microwave oven HIER, violent boiling frequently occurs, and it is often necessary to refill the slide incubation containers (Coplins jars) several times and repeat the microwave heating in order to obtain satisfactory HIER. In contrast, boiling of the HIER solution does not occur within a pressure cooker, so there is no need to refill the Coplin jars during the HIER procedure. Because of the boiling that occurs in a microwave, the maximum temperature that can generally be achieved in a microwave is 100 degrees C, whereas a pressure cooker at 15 psi generally obtains temperatures of about 120 degrees C. The higher temperatures in a pressure cooker can allow for overall shorter HIER incubation times. In my laboratory, we have had much better results with pressure cooker HIER than microwave HIER, so we use a pressure cooker for the vast majority of our HIER. An additional advantage of pressure cooker HIER was that our sensitivity increased substantially, so that we were able to dilute many of our primary antibodies by a factor of 4 to 8 compared to the dilutions we used for microwave HIER, with equivalent or superior results.

The amount of pressure-cooking time is important, and a 2 or 3-minute variation in pressure-cooking time may have a substantial impact on the end result. It is recommended that each laboratory determine its own optimal pressure-cooking time. Some laboratories use as short as 2 minutes, although in our laboratory we found that a pressure cooking time of 9 minutes (using a standard household model with a hotplate) gave us the best overall results for a wide variety of antigens. Timing is started when the top pressure vent release cover begins to rock back and forth, indicating that full pressure has been reached. Additionally, it is important to keep the cool down time consistent, since variation in the cool down time will cause variation in results. In addition to standard household pressure cookers heated on electric hot plates, a number of manufactures also market electric pressure cookers with built-in timers, which may be more convenient to use for some laboratories. We currently use these electric pressure cookers (BioCare, Walnut Creek, CA) in our laboratory, and with the particular model that we use, we turn the dial on the timer past the 20 minute mark and back to 5 minutes (so the slides are pressure-cooked at full pressure for 5 minutes). Simultaneously, we set a separate timer for 48 minutes, and when the 48 min. timer goes off, the slides are removed from pressure cooker. This allows for a substantial cool down time and also ensures consistent results from day-to-day.

Other Heat Sources for HIER

There are some laboratories that have the luxury of having an autoclave available for HIER, and I expect that autoclaves would behave very similar to pressure cookers. Other laboratories prefer vegetable steamers as a heat source, and these are inexpensive and avoid many of the problems of uneven heating suffered by microwave ovens. However, they do not allow the attainment of temperatures as high as those in pressure cookers, and when comparing steam heat with pressure cooker HIER in my laboratory, pressure-cooking is superior except for a small number of antigens (HMB-45, MOC-31 and mesothelin). Hot water baths are also used by some laboratories with good results (23). Another group reported a "low temperature heat-mediated antigen retrieval" method using 0.2M boric acid (pH 7) or 50mM Tris/2mMEDTA (pH 9) at 60

degrees C (32a), although this method required 16 hours of antigen retrieval for satisfactory immunostaining, a luxury that may not be available in many laboratories, including my own.

Epitope Retrieval with Ultrasound (Sonication-induced epitope retrieval, SIER)

In 1996 Portiansky and Gimeno (33) reported the use of an ultrasonic cell disrupter for epitope retrieval, and in their laboratory found it to be superior to microwave and pressure cooker HIER for the detection of cytokeratins 13 and 16. Shortly after reading this paper, I leased an ultrasonic disrupter (since they cost about \$3000) identical to that used by the authors, and had very disappointing results. Our pressure cooker method was far superior, both from the standpoint of tissue adhesion and effectiveness of epitope retrieval. Failure of ultrasonic vibration for epitope retrieval has been confirmed by Hammoud and Van Noorden (33a). Brynes and colleagues (34) described a method for cyclin D-1 staining employing microwave HIER followed by SIER in an 80 watt ultrasonic cleaner, and they found this method superior to microwave HIER alone. (We use our standard pressure-cooker HIER for cyclin D-1 with very good results).

Effect of HIER on Endogenous Biotin in Tissues

In addition to enhancing the detectability of epitopes, HIER has also been found to enhance the reactivity of any endogenous biotin present in the tissue being studied. When employing avidin-biotin based detection systems, endogenous biotin activity may be responsible for unwanted bothersome artifacts (35-37), secondary to binding of the avidin in the detection complex to endogenous biotin within the tissue. Failure to recognize this artifact has been responsible for some embarrassing erroneous publications in the immunohistochemistry literature (38-39). Pyruvate carboxylase, an enzyme in the Krebs cycle, contains four molecules of biotin, and is localized in mitochondria within the cytoplasm of cells. Considering this fact, it is not surprising that endogenous biotin artifact is most prominent in metabolically active cells that contain numerous mitochondria, such as liver and kidney cells. As one can readily surmise, oncocytomas (which are packed with mitochondria) show the most striking and intense endogenous biotin artifacts, although I have seen significant endogenous biotin artifact in many other tumor types, including carcinomas of the lung, breast, prostate, and thyroid. A clue to recognizing this artifact is that the granular cytoplasmic reactivity appears "muddy", and there is a lack of cell-to-cell heterogeneity with this artifact. However, the distribution of the chromogen in the cells can vary, with some cells showing a uniform distribution of the chromogen, whereas others may show a distinct polarity of this artifact, with the granular chromogen deposition located preferentially near one side of the cell.

Because this artifact can be extremely intense and is often precisely localized to tumor cells, **it is important to always subject the negative control slides to the same HIER procedures as the slides treated with primary antibody.** If the negative control slide is not subjected to HIER, it likely will not show significant endogenous biotin artifact, causing a high risk of misinterpretation of endogenous biotin activity as true immunoreactivity by the unwary.

Endogenous biotin activity shows variable degrees of enhancement depending on the particular HIER solution used. In our laboratory, stronger artifacts are observed with Tris buffer and urea HIER solutions, with lesser degrees of this artifact observed when citrate buffer is used for HIER. Fortunately, this artifact is easy to overcome, and blocking of endogenous biotin activity will be discussed in the "analytic variables" portion of this presentation.

Formalin Substitutes

Due to the antigen masking effects of formalin, there has been some interest in using formalin substitutes for primary fixation (40-43), and this has spurred a number of manufactures to market a variety of formalin substitutes. These substitutes have received somewhat mixed reviews in the literature. There are basically two categories of formalin substitutes; aqueous-based preparations and alcohol-based preparations. The aqueous-based preparations do not really function as fixatives per se, but rather as tissue preservatives that prevent autolysis. Fixation of the tissue actually occurs once the material is placed in the tissue processor (where it is fixed from formalin or alcohol in the processor). I have tried a number of the substitutes in my laboratory, and I cannot enthusiastically endorse any of them, mainly because all of them that I have tried cause noticeable cell shrinkage, and the morphology (at least in my opinion) is inferior to properly handled neutral buffered formalin-fixed material. Although some of these formalin substitutes have the advantage of requiring little or no antigen retrieval, in many instances the use of appropriate epitope retrieval procedures allows immunostains on formalin-fixed paraffin sections to match or surpass those obtained with these formalin substitutes.

Avoiding Overfixation in Formalin, and Suggested Fixative for Prolonged Tissue Storage

As discussed above, it is important to avoid overfixation in formalin to prevent undue antigen damage, which can easily occur if tissues are allowed to sit in formalin over a weekend or longer. In order to avoid this problem, tissues that will not be processed for a long period of time should be placed in 70% alcohol for long-term storage, since this will minimize adverse effects of antigen damage. If one is using an automated tissue processor, it is possible to program many of these processors so that the "holding time" that occurs during a weekend (before the processor starts on Sunday night) occurs in alcohol rather than formalin.

Other Fixatives

B5 is a commonly used mercury and formalin-based fixative, particularly for lymph node and bone marrow pathology, since it provides excellent nuclear morphology. Like formalin, B5 also induces molecular cross-linking, but to a greater extent than formalin. Although there are some authors who prefer B5 to formalin for immunostaining purposes, there are a number of important antigens that do not perform nearly as well in B5 as in formalin, particularly CD5 (clone 4C7) (44). We have also noted that CD30 and CD23 perform substantially better in formalin-fixed tissue. For this reason, my personal fixative preference for immunophenotyping lymph nodes and bone marrows (as well as virtually everything else) is neutral buffered formalin. Most other antigens perform satisfactorily in B5, and a few even perform better in B5 than formalin (such as kappa and lambda light chains). **Zenker's** is also a mercury-containing fixative, and in my experience it performs similar to B5. "De-Zenkerization" is necessary to remove precipitated pigment when using these fixatives, and it is important to realize that this de-Zenkerization step should NOT be performed prior to the antibody incubations, but should be performed immediately before the counterstain is applied, at the end of the immunostaining procedure. This observation was confirmed and reported by others in 2000 (44a), and these authors found that the Lugol's iodine is the offending agent.

Bouin's fixative is a formalin-based solution with picric acid (an explosive compound) and acetic acid, and is preferred by some pathologists for certain types of specimens because it provides excellent cytologic detail. To my knowledge there are no large studies evaluating the effects of Bouin's fixation on a wide range of antibodies, although in my laboratory I have

observed severe deleterious effects on some immunostains, particularly CD5 (clone 4C7), CD10 (CALLA, clone 56/C6), and cyclin D-1 (clone AM29). For this reason, I avoid it whenever I can.

Hollande's fixative is a favorite of some gastrointestinal pathologists, although I have personally never employed this fixative routinely. However, I have received many referral cases that have been fixed in this solution (usually gastric biopsies to rule out lymphoma), and it has caused severe adverse effects on a number of lymphoid-related antigens, so I detest this fixative. In fact, in several of my referral cases, the use of this fixative has hindered optimal patient care, since an inability to completely immunophenotype abnormal lymphoid infiltrates has necessitated re-biopsy in several patients. I think I might be correct in saying that the gastrointestinal pathologists who prefer Hollande's fixative are not responsible for immunophenotyping their gastrointestinal lymphomas!

Zinc formalin is prepared by the addition of various zinc salts to formalin, and this fixative gives very good nuclear morphology while slowing the cross-linking effects of formalin, so it is advocated by a number of authors as a superior alternative to formalin (45-47). In my laboratory, I have not noticed any marked adverse effects of zinc formalin on the detection of common antigens, and for this reason I would prefer zinc formalin over B5, for those who desire the improved nuclear morphology associated with these fixatives. Some laboratories have found that CD5 (4C7) and CD10 (56C6) perform much better in zinc formalin than in regular formalin (Marsha Price, personal communication). Some of our clients use zinc formalin, and one thing that we have noticed is that small biopsies fixed in zinc formalin do not tend to adhere to slides as well during immunostaining as material fixed in neutral buffered formalin.

ANALYTIC (STAINING) VARIABLES

Having discussed "pre-stain" variables, attention will now be directed toward each of the various steps in the immunohistochemistry staining procedure. Comments on each of the steps will be made, to share my personal experiences (and biases) regarding alterations to these steps that have given us best results in our laboratory. Again, since every lab is different, my way is not necessarily the best way for everyone. Although one can certainly argue that there is no true "standard" method for IHC, for the purposes of discussion, at this point I believe it is useful to briefly review the various steps in a common "standard" method. This so-called "standard" method for paraffin sections is the one that I learned in 1980 when I first began performing my own immunostains at the bench. Since avidin-biotin methods are by far the most commonly used methods for IHC, most of the discussion will relate to avidin-biotin based techniques.

(So-called) STANDARD IHC METHOD

1. Cut paraffin sections and mount on adhesive slides.
2. Deparaffinize slides in xylene and graded alcohols to water.
3. Quench endogenous peroxidase activity. Rinse in buffer.
4. Incubate with "blocking serum". Rinse in buffer.
5. Perform epitope retrieval step (antigen retrieval or protease) if needed. Rinse in buffer.
6. Apply primary antibody, incubate, and rinse in buffer.
7. Apply secondary (link) antibody, incubate, and rinse in buffer.
8. Apply detection complex, incubate, and rinse in buffer.
9. Develop reaction product with chromogen, counterstain, dehydrate, coverslip, and view.

Tissue adhesives

All immunohistochemists know that tissue adhesives are required to maintain adhesion of the diagnostic material on the slides. Another point to keep in mind is that poorly fixed tissue will not stick to the slides, regardless of what adhesive is used. A number of adhesives have been tried over the years (dilute Elmer's blue, chrom alum, etc.), but in general the most effective adhesive slides have been coated with poly-L-lysine or silane. Adhesive slides are available from numerous manufacturers, although we have had best results by preparing our own silanized slides, based on a previously published method (48). On difficult cases, we have had good results by taking our routine silanized slides, and "double dipping" them a second time in the silane adhesive solution. These "double dipped" silanized slides have been far more effective than any of the numerous commercially-prepared adhesive slides that we have evaluated.

Drying of slides

There are a number of options available for drying of slides, depending upon how much time one desires to devote to this step. Drying the slides in a 56-60°C oven for 1 hour works well, and if you have the luxury of drying your slides overnight, this is even better. For those who need a more rapid turnaround time, a microwave oven can be used to dry slides, and this is the method that we currently use in my laboratory (3-4 minutes on "high"). We have also used a small household convection oven with satisfactory results, although drying takes a bit longer. Some authors have described the use of drying sections on a hot plate with satisfactory results (49), although this method does not lend itself well to processing large numbers of slides simultaneously, as with the other methods described above.

Recently, one group reported that the drying and deparaffinization steps may be omitted (50), discussed in the following paragraph under "deparaffinization".

Deparaffinization

Xylene is a commonly used deparaffinizing agent, although some investigators have found that xylene damages certain antigens (Dr. Mark Wick, personal communication), and as a result, some immunohistochemists prefer xylene substitutes. We have not noted major deleterious effects of xylene, and prefer xylene over substitutes, since xylene seems to be more effective at complete deparaffinization than the various substitutes that are currently marketed.

In 1997, Yorukoglu and Congoz (50) reported that the slide drying and deparaffinization steps can be omitted, by placing the slides immediately after cutting into Coplin jars of buffer and heating in a microwave oven for 5 minutes, along with a second Coplin jar filled with buffer (but lacking slides). The slides are then transferred to the second Coplin jar (now containing hot buffer), microwaved for another 5 minutes, and allowed to sit in the hot buffer for another 15 minutes. At this point, the slides are then immunostained as usual. This procedure essentially involves omitting the drying and deparaffinization step, and proceeding directly to the epitope retrieval step, thereby saving time. There are a number of reagent vendors that have picked up on this trick, and are currently marketing solutions for this purpose. We have tried this technique on a number of occasions, and indeed it does work well, although we have noticed artifactual small nuclear bubbles when employing some (but not all) of these reagents. In our hands, this technique has not saved a great deal of time, and can be fairly expensive if commercially purchased solutions are employed. Some laboratories employ dilute soap (1% Softsoap) solutions for this purpose with good results (Marcia Price, personal communication).

De-Zenkerization (WAIT UNTIL LATER!)

We found out the hard way that de-Zenkerization of B5-fixed or Zenker's fixed slides should NOT be performed until the very end of the staining procedure, as performing this step before application of the primary antibody has shown marked deleterious effects on the detectability of many antigens. For this reason, I suggest that the step be performed immediately before the counterstain is applied, near the end of the staining procedure.

Heat-Induced Epitope Retrieval (HIER) or Protease Digestion

As previously discussed, optimal staining for many epitopes requires that HIER or protease digestion be performed before application of the primary antibody. Details of these procedures have been previously discussed above.

Quenching of endogenous peroxidase

There are a wide variety of methods that may be used to quench endogenous peroxidase, which is a necessary step in methods that employ a peroxidase-based detection system. If this is not performed, cells that have abundant endogenous peroxidase (such as red blood cells or neutrophils) will show chromogen deposition, potentially interfering with accurate interpretation. My own personal favorite method of quenching endogenous peroxidase was initially reported by Li et al (51), and involves a 10 min. incubation with 0.3% H₂O₂ in phosphate buffered saline (PBS) with 0.1% sodium azide added. (We have found that the solution works best at room temperature, and the use of colder solutions requires longer incubation times for the equivalent quenching effectiveness). This is a relatively "gentle" technique that does not damage some fragile antigens that may be adversely affected by other techniques. It does not completely quench peroxidase activity in eosinophils, however, but that is usually not a major problem in interpretation, since eosinophils are so readily recognized. In those instances where a "stronger" method of peroxidase quenching is required (particularly when employing tyramine enhancement techniques discussed below), we have had good results using warm 3% H₂O₂ as a quenching solution, although this does damage some antigens to a noticeably greater degree than when employing Li's method. In this situation, we heat 3% H₂O₂ to about 70 degrees C in a microwave oven (50 mls for 30 seconds on "high") and incubate the slides for 1 minute in this solution.

Although most laboratories perform this quenching step before the application of the primary antibody, there is some evidence that suggests that better results may be obtained by performing this step after the link antibody incubation (52), rather than before the primary antibody incubation period.

Blocking serum steps

There are many laboratories that employ a step with blocking serum, prior to incubation with primary antibody. In many cases, this blocking serum consists of dilute serum from the same species used for production of the link antibody, and theoretically this step will decrease subsequent nonspecific binding of the link antibody. Personally, I have never been impressed with the performance of these blocking reagents, and when I have compared stains with and without blocking serum steps, I have usually found better and cleaner results when the blocking serum steps are omitted. This has been particularly true when employing the high-quality primary and secondary antibody reagents that are now available, and for that reason we do not routinely perform a blocking serum step in our method. Dilute solutions of casein (a protein found in milk) have also been used by some laboratories to decrease nonspecific staining (53). Additionally,

there are some laboratories that add dilute serum or dilute casein to their antibody diluent solutions or their rinse buffer solutions, to theoretically reduce background staining.

Blocking of endogenous biotin in tissues

Endogenous biotin artifact usually appears as granular cytoplasmic reactivity in cells, and is particularly noticeable in liver cells and kidney proximal tubules, although it also may be substantial in tumor cells of widely varying types. Since the widespread use of heat induced epitope retrieval (HIER) techniques, endogenous biotin has become a much more serious problem that needs to be dealt with whenever avidin-biotin detection systems are employed. This is due to the fact that HIER techniques markedly enhance the detectability of any endogenous biotin present in the tissue, just as they may enhance the detectability of the antigen of interest. This artifact can be extremely intense (35), and may be exquisitely localized to tumor cells, with "crystal clean" background stroma. Indeed, failure to recognize this artifact has been responsible for the publication of erroneous literature (38-39). For this reason, it is my opinion that **any laboratory employing HIER techniques should routinely include steps for blocking of endogenous biotin in any immunostain that is subjected to HIER**. An additional important point to keep in mind is that it is critical that negative control slides also be subjected to the same HIER procedures that are used for antibody stains during the staining run, since only then will any endogenous biotin activity be apparent on the negative control specimen.

Blocking of endogenous biotin may be accomplished by several methods, and steps taken to block endogenous biotin should be performed immediately after any HIER or protease digestion steps, and before the incubation with primary antibody (although theoretically they could be performed immediately after the primary antibody incubation and before the link antibody incubation). This can be performed by incubating the sections (at room temperature) for 20-30 minutes with 0.05% free avidin in PBS (0.001% to 0.1% solutions are reported to work) (54), and this avidin will bind to any endogenous biotin present in the tissue. This is then followed by a 15 minute incubation (at room temperature) in 0.05-0.2% biotin in PBS (concentrations as low as 0.001% are reported to be effective), which will saturate any remaining biotin-binding sites. Many commercial reagent suppliers sell solutions for blocking of endogenous biotin, and they perform satisfactorily.

Shortly after we began employing pressure cooker HIER routinely, we began to notice significant endogenous biotin artifact in many different tumors, and realized that we needed to incorporate a routine step for blocking of any endogenous biotin in our method. We initially started with commercial reagents, but quickly realized that they are quite expensive. We decided to make our own reagents, but were shocked when we found that avidin costs about \$3400 per gram. Knowing that the source of avidin is egg whites, we postulated that dilute egg white solutions may be useful as an inexpensive source of avidin, and indeed dilute egg white solution (2 egg whites diluted to 200 mls with distilled water, preceded by a distilled water rinse) works very well as a source of avidin (35). Furthermore, when we discovered that milk is a rich source of biotin, we showed that skim milk may be effectively substituted for commercial biotin in endogenous biotin-blocking procedures (55). However, after employing skim milk as a source of biotin successfully for several years, we began to notice an unusual artifact that we eventually traced to a problem with the milk (perhaps the problem was related to some change in the cow's diet?). We encountered this artifact when we would attempt to perform stains using an antibody that was freshly diluted from stock (most commonly when we were titrating a new antibody), and we would experience widespread and severe problems with background staining. Interestingly, we did not experience this artifact with our other stains, and if we let the antibody solution "age" for five days or more, we would not experience this artifact. Initially I had thought that perhaps

the artifact was due to drying or due to a problem with our antibody diluent solution, although we were able to reproduce this artifact using commercially purchased antibody diluents. When we performed parallel stains employing an avidin-biotin-free detection system (Dako Envision), we did not experience this artifact. At this point, we knew that it had to be somehow involved with the avidin biotin interaction, and upon eliminating skim milk and returning to 0.2% biotin in PBS (or reconstituted powdered dried milk) as a source of biotin for blocking procedures, the artifact disappeared. **Therefore, I no longer recommend regular skim milk as a source of biotin for endogenous biotin blocking procedures, but rather recommend using 0.2% biotin in PBS or reconstituted powdered dried milk (5% dried milk solution. i.e., 25 grams dried milk in 500 ml PBS/Tween buffer) for this purpose.** We still employ dilute egg whites as an avidin source with great success. Therefore, in our laboratory, we perform blocking of any endogenous biotin by incubating the slides in dilute egg white solution (as a source of avidin), followed by a rinse in distilled water and then an incubation in either 5% dried milk solution or 0.2% biotin in PBS. This has worked extremely well for us, and has also had the benefit of decreasing nonspecific background.

Choice of Diluent for Antibody Solutions

There are a number of different solutions that are used by laboratories for diluting primary and secondary antibodies. In general, the solutions consist of buffer with a stabilizing agent added, which in many laboratories consists of bovine serum albumin (BSA), or some similar protein. There is some literature stating that antibody solutions containing less than 50 mg/ml protein may be unstable at four degrees C for two weeks (56-57). Although for many years we used PBS with 0.2% BSA as our diluent, we found that increasing the BSA concentration to 1% improved our results somewhat, so currently we employ PBS with 1% BSA as our antibody diluent. For a time, we used concentrations of BSA as high as 5%, but at this concentration, we had occasional problems with strong nonspecific background staining, when using our adhesive slides prepared in-house. We also add a small amount (0.1%) of Tween 20 (a detergent) to our buffer, since this assists in uniform spreading of the reagents. It is also advisable to include a small amount of some type of antimicrobial agent (thimerosal or similar agent) to prevent microbial contamination. In general, we try to maintain the pH of our diluent near 7.4.

There is some recent evidence that suggests that the performance of monoclonal antibodies can vary with respect to the ion content and pH of the antibody diluent used (58). The author of this work suggests that the optimal primary antibody diluent is 0.05M Tris buffer [(Trizma Base Tris(hydroxymethyl)aminomethane, Sigma T-1503] with 15 mM sodium azide (Sigma S-8032). Furthermore, since this author found that different monoclonal antibodies respond best to dilutions at different pH's, he recommends that all new primary monoclonal antibodies be tested with this diluent at pH 6.0 and also pH 8.6 (prepared by adding concentrated HCL to 0.05M Tris buffer until the required pH is obtained.). At the time of this writing, we have not had the opportunity to evaluate these diluent buffers in our laboratory.

Buffer Rinsing Steps

Thorough and effective rinsing is important between the various steps of the procedure, and most laboratories employ either PBS or Tris-buffered saline (TBS) as a rinse buffer. As mentioned above, we find very helpful to add Tween 20 to our rinse buffer, since this aids in effective rinsing. Although previously I never thought of using anything else for rinsing steps, a conversation with another immunohistochemist (Dave Tacha, BioCare, Walnut Creek, CA) led me to try distilled water with Tween 20 (DW/Tween20) rather than PBS for the rinsing steps. When we evaluated the performance of DW/Tween 20 for our rinsing solution between steps, it

performed just as well as using PBS with Tween 20, so we now routinely use DW with 0.2% Tween 20 (2 ml Tween 20 in 10 liters of DW) for all of our rinses that previously used buffer. This has saved us substantial amounts of reagent cost as well as reagent preparation time, since preparing DW/Tween 20 is far easier and cheaper than preparing our previous PBS buffer rinsing solutions.

Primary Antibody Incubation

Obviously the primary antibody incubation is an important step in any immunostain, and since the stain is only as good as its worst component, starting with a high quality antibody is very important. A crucial point to realize is that **THERE IS NO SUCH THING AS A "PREDILUTE READY-TO-USE" ANTIBODY**, regardless of what the manufacturer will tell you. I cannot stress this point enough, since I know that blind acceptance of this fallacy is responsible for many problems in both neophyte and established laboratories. Another key point is to **NEVER BELIEVE THE MANUFACTURER'S RECOMMENDATIONS FOR TITER OF THE PRIMARY ANTIBODY**. Occasionally they will be correct in their suggestions, but more often than not the titer that they will suggest is not optimal for the conditions in your laboratory. Sometimes the suggested titer will be way off the mark. Probably the most glaring example of this is a polyclonal antibody that we use in our laboratory at a titer of 1:50,000 in which the suggested titer was 1:200. At 1:200, absolutely nothing stained. From the foregoing it can be surmised that **IT IS ESSENTIAL FOR EVERY LABORATORY TO DETERMINE THE OPTIMAL TITERS OF ALL PRIMARY ANTIBODIES BEFORE PUTTING THEM INTO DIAGNOSTIC USE**. In my opinion, failure to do this constitutes "immunohistochemical malpractice". Each new lot of antibody should also be titered, as occasionally there is some variation in optimal titer from lot to lot.

Titering of Antibodies

Titering of antibodies is probably one of the most confusing aspects of IHC for people just learning the technique, and I believe that the failure to determine optimal titers is the main reason for frustrating results (or perpetual mediocrity) experienced by many laboratories. In order to assist those who may not be performing this essential function routinely, I am including an appendix on "Titration Studies in Immunohistochemistry" at the end of the handout. Determining optimal titers is not difficult, but it involves some work and also awareness of the features of a "true-positive" and a "false-positive" IHC stain.

The first step in determining optimal titers is to decide on a series of titers to try. (As discussed below, if working with a new antibody, it is usually best to attempt to determine which epitope retrieval method is best before determining the optimal titer). As a general guideline, I will usually do the first titer at the level suggested by the manufacturer, and then test a series of further 2-fold dilutions from there. For example, if the manufacturer suggests a titer of 1:50, my first set of titer studies will include stains done with the primary antibody diluted at titers of 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600. Usually an appropriate titer can be chosen from the first set of studies, but not always, and one may need to try either more dilute or more concentrated antibody titers before finding the best one. You may be amazed at some of the optimal titers that you may find. For example, at one time the polyclonal anti-lambda antibody that we used in our laboratory for detecting lambda-positive plasma cells was used at a titer of 1:2,000,000 (That's right, 1 to 2 million!!)

Next, one must choose what type of tissue to use for the antibody titration studies. Many laboratories simply choose a case known to be positive for the antigen in question and perform

the titration studies on sections from this one case. For example, sections from a known melanoma are selected to determine the optimal titer of HMB-45, a melanoma-associated antigen. At first glance this may seem logical, but in reality this approach is woefully inadequate, and in practice it can be overtly dangerous, since it does not assure that you will be choosing the appropriate titer. It's obviously important to assure that a known positive case stains appropriately, but what about other positive cases (that may have greater or lesser antigen densities)? Additionally, what about cases that should be negative? **It is just as important to assure that known negative cases are indeed nonreactive for the antigen in question.**

To address these issues, there are several approaches. One is to do a series of titration studies on a number of different known positive cases as well as a number of known negative cases. Although I do know of some labs that adopt this approach, I know for a fact that very few laboratories will go through this much work to titer an antibody. Another approach (used in our laboratory) is to employ multitumor sandwich block controls for the purpose of titrating antibodies (59). The preparations that I currently use contain 80 tumors of all different types (carcinomas, neuroendocrine tumors, sarcomas, melanomas, lymphomas, etc.), mounted in a well organized grid-like fashion in a size that is easily mounted on one half of a microscopic slide. This allows a wide variety of known positive cases (of varying antigen density) and a wide variety of known negative cases to be assessed very quickly and efficiently, without consuming much time or reagent. As such, when I perform a series of titration stains, I select a known positive case and mount the sections from this case on the same slides with sections from my multitissue sandwich control block. These slides are then used for the determination of optimal titers. In my mind, this is the best way to assess the optimal titer of a primary antibody.

True-positive vs. False-Positive Staining

Having performed the titration studies, they then must be assessed. In order to evaluate them appropriately, one must be aware of the characteristics of a **true-positive stain** and a **false-positive stain**. A "true positive" stain shows chromogen deposition in cells or structures that truly contain the antigen of interest. In contrast, a "false positive" stain is one where the chromogen is localized to cells or structures that in reality lack the antigen of interest. When using diaminobenzidine (DAB) chromogen for peroxidase-based detection systems, this problem can be expressed simply as "all that is brown is not real". Stains in which the entire section (both tumor cells and intervening non-neoplastic stroma) is a dark "muddy" brown are easily recognized by nearly any observer as falsely positive. Fewer observers realize that false-positive staining commonly occurs in a different situation: where the tumor cells are strongly stained but the background non-neoplastic stroma is "clean" and free of chromogen deposition.

Before discussing this latter type of false positive staining (where the chromogen is paradoxically exquisitely localized to the cells of interest), I believe that it is useful to keep in mind the patterns of reactivity seen with true positive stains. One feature characteristic of true-positive stains is **cell-to-cell heterogeneity**. This refers to the fact that the deposition of the reaction product often varies in intensity among cells, and also often within parts of a single cell. Another pattern of true positive immunoreactivity is that of **distinct crisp cell membrane reactivity**, which is only rarely seen in false-positive staining artifacts. Immunoreactivity restricted to **cell nuclei** (e.g., estrogen receptor) is another type of true-positive reactivity, but in a true-positive nuclear stain one often can still appreciate some degree of cell-to-cell heterogeneity. A potential trap in interpretation of nuclear staining is spurious reactivity of "optically clear" nuclei described in gestational endometrium (and also in some pulmonary blastomas), secondary to binding of endogenous biotin present in these nuclei (60). I have also seen artifactual false-positive staining of cell nuclei in some cases (particularly with CD15) in areas adjacent to

necrotic tissue, but in these instances the intensity of the label is uniform and often somewhat indistinct (i.e., it lacks cell-to-cell heterogeneity and may appear "muddy"). On occasion a false positive nuclear stain may also be seen when heavy-metal fixatives are used, such as B-5 or Zenker's, and I have also seen intensely positive false positive nuclear staining artifacts when employing strong acids as decalcifying agents or as epitope retrieval solutions, the latter finding also reported by others (61). For this reason it is important to know the expected pattern of immunoreactivity (nuclear, cytoplasmic, or membrane) for the antigen of interest. False positive staining of nucleoli has also been reported (62) with CD20 (L26), and indeed I have seen this pattern many times in my own cases. I have also frequently seen nucleolar artifact with CD7 immunostains.

An important trap alluded to above is that false-positive immunoreactivity may be very intense and can be precisely localized to the cells of interest (usually tumor cells), with no staining of the background stroma observed. However, this type of reactivity can be readily recognized if the observer is aware that this type of false-positive staining characteristically LACKS cell-to-cell heterogeneity. In these instances, the cells display a uniform cytoplasmic "blush" that appears "muddy" and indistinct, lacking the crisp heterogeneous quality of a true positive stain. This pattern of false positive staining is usually secondary to the use of an inappropriately concentrated titer of primary antibody, or to using a (so-called) "predilute ready-to-use" antibody at an inappropriate titer (i.e., used as supplied without testing further titers).

Binding of avidin components to endogenous biotin present in tissues or cells may also cause false positive staining when avidin or streptavidin-based detection systems are used. As previously discussed, this is most frequently observed in liver and kidney (since these tissues have numerous mitochondria and therefore a high biotin content), where it appears as granular cytoplasmic chromogen deposition that is noticeable on the negative control specimen as well as on the patient specimen (**provided that the negative control was subjected to the same epitope retrieval procedures**). As discussed previously, this problem can be economically and effectively dealt with by routinely employing steps to block endogenous biotin activity in the staining method, using dilute egg whites as a source of avidin and reconstituted 5% dried milk or 0.2% biotin in PBS as sources of biotin (35, 55) (see "Blocking of Endogenous Biotin Activity in Tissues" above). At this point the primary antibody may then be applied.

Conditions of Antibody and Detection Complex Incubations

The conditions used for antibody and detection complex incubations also vary widely from laboratory to laboratory, and include variations in incubation times, temperatures, and conditions. When I initially learned the technique in 1980 as a pathology resident at the University of Minnesota, the "gold standard" involved incubation of the primary antibody overnight in flat incubation trays placed in the refrigerator, followed the next day by one-hour incubations at room temperature with link antibody and detection complex. Indeed, this works well, although it does not allow a run of stains to be completed in a single day, a distinct disadvantage in the diagnostic setting. Since that time, we have discovered that there are a number of alterations that can be made that will substantially decrease incubation times needed for optimal stains. Chief among these alterations is the use of mixing of reagents during the incubation periods, a feature also employed by several automated immunostainers. We perform this mixing of reagents by using an orbital rotator set at 40 rpm, and place the incubation trays on top of this rotator during incubations (63). By doing this, we were able to shorten our incubation times to 30 minutes with primary antibody, and 10 minutes with both link antibody and detection complex, and yet achieve stronger and cleaner stains than we obtained when using the prolonged incubation periods described above. If using this technique with flat slide incubation trays, it is critical that the slide

trays remain level during the incubations, or areas of the tissue sections will dry out during the incubation, resulting in poor stains.

The temperature of antibody incubation can also be varied. Some investigators prefer to perform incubations in the refrigerator, whereas others employ room temperature or 37°C. Increasing the temperature tends to speed the antigen antibody reaction, although there are some antibodies that apparently do not perform as well at higher temperatures. I am not aware of published systematic studies evaluating the effect of antibody incubation temperature on immunostain results, although some immunohistochemists who have evaluated this in their own laboratory have discovered that overall, optimal results appear to be achieved when employing incubation temperatures of around 25°C (Dave Tacha, BioCare, Walnut Creek, CA, personal communication). Prior to becoming aware of Tacha's results, we performed all of our antibody incubations at 37°C. We now perform them at 25°C with similar results, and somewhat fewer problems with slides drying out during the incubation procedures

"Batch" or "Bulk" Incubation Staining Using Bulk Reagents

Many laboratories that employ manual methods use flat incubation trays, and perform antibody incubations and buffer rinses by manually manipulating the slides one at a time. On a busy day, this is a very time-consuming operation, and manual manipulation of large numbers of slides begs for errors to be made. An alternative approach first reported in 1982 and subsequently by a number of other laboratories (64-67), involves the repeated reuse of antibody and detection complex reagents in a batch format. In this method appropriate dilutions of antibody and detection complex reagents are placed in large volume containers, and the slides are simply immersed into the containers to perform the incubations. When I first became aware of batch methods of immunostaining, my skepticism prevented me from trying them, since I was afraid that the antibody reagents would deteriorate rapidly if maintained in dilute format and repeatedly reused. I also had concerns about contamination of antibody solutions, both by other antibodies and by microbes. However, after speaking with a technologist in 1995 who had used this method successfully, I decided to give it a try, and I have been a strong proponent of this method ever since. In addition to saving both time and money, this technique is far easier for the technologists to perform, and nearly eliminates problems with slides drying during the incubation and rinsing steps, since the slides are completely immersed in reagents, rather than having reagents dropped onto the surface of the slide. I have been amazed at how many immunostains can be performed using the same diluted reagents. As an example, in our laboratory we have used one diluted batch of monoclonal link antibody to process at least 50,000 immunostains, and have only had to add fresh reagents to the containers to keep the levels of reagents high enough in the container to cover the tissue mounted on the slides completely.

Depending on the volume of stains processed in a laboratory, the containers used will obviously vary. In our laboratory, for antibodies that are commonly ordered, we place appropriate dilutions of the primary antibodies into 5-slide capacity plastic slide mailing containers, and store them vertically in racks in the refrigerator. Slides are simply immersed in these plastic slide containers to perform the appropriate primary antibody incubations. For antibodies that are not frequently ordered, we continue to perform the primary antibody incubation in a flat slide tray. After the primary antibody incubation is completed, the slides are removed from the containers (or slide trays), and they are subsequently given a "squirt rinse" with rinsing solution. Since the remainder of the staining technique is identical for all stains, the slides can then be transferred into large capacity racks, which are subsequently immersed in large volume containers of appropriately diluted reagents (link antibody, detection complex, rinsing solution, etc.). This dramatically decreases the amount of individual slide manipulation that must

be performed by the technologist, which substantially increases the volume of slides that may be processed by a particular laboratory. Since reagents are continually reused, reagent costs are also minimized.

Obviously, between each of the antibody incubation steps, thorough rinsing and draining of the racks of slides is important. For rinsing purposes, the racks are placed into three changes of fresh rinsing solution, and then the racks are tilted on a paper blotting towel to allow the rinsing solution to drain off as thoroughly as possible without any drying of the tissue sections on the slides. As discussed above, since we employ distilled water with Tween 20 as our rinsing solution (rather than buffer), it is very simple and economical to prepare large volumes of this rinsing solution for use in large volume staining situations.

One critical point to keep in mind if using this technique with a peroxidase-based detection system is to make certain that the detection complex is diluted in a peroxidase-stabilizing buffer solution, as this is key to the success of this batch method involving repeated reuse of reagents. These peroxidase-stabilizing buffers are available in concentrated format from a number of manufacturers.

Another practical point to keep in mind is that the starting temperatures of the batch reagents should be kept consistent. We store our reagents in the refrigerator overnight, but they are allowed to reach room temperature before use, since if the temperature varies from day-to-day, the staining results will also vary.

Over time, certain reagents may weaken somewhat, and in our experience, the first reagent that tends to lose activity is the streptavidin-HRP detection complex. This problem can be recognized by noticing a slight weakening in sensitivity for all stains over the course of several consecutive days. When this occurs, we simply add one or more vials of concentrated detection complex reagent to the batch, and sensitivity is restored to the prior level. Parenthetically, we have not noticed this weakening sensitivity problem (processing up to 35,000 stains through a single batch at the time of this writing) of a non-avidin-biotin polymer-based detection system (PowerVision, ImmunoVision Technologies, Daly City, CA) (84) that we began using for a period of time before this writing. Using this reagent, up to this point it has only been necessary to add fresh reagent as the level in the containers decreases, and sensitivity has not noticeably decreased with extended and repeated reuse. I am sure that eventually this reagent will substantially deteriorate with continued use, but it has not occurred yet!

If a particular antibody stain appears to be weak when other stains are satisfactory, it may be necessary to "spike" the dilute primary antibody solution with an appropriate amount of concentrated antibody, to restore the sensitivity of this particular antibody stain to its desired level.

It is also worthwhile to record the starting pH of batch solutions and monitor the pH of the link antibody and detection complex solutions as they are used, as in many cases over the course of time the pH will drift, usually down. We routinely check the pH on these batch solutions twice a month, and adjust them to the starting pH of the fresh solutions

We have found this batch manual method to be so economical and efficient that we currently do not see any advantage in automating our laboratory using currently available instruments marketed for this purpose, either from a workflow or economic standpoint, despite our situation as a relatively high volume referral IHC laboratory.

Secondary (Link) Antibody Incubation

There are several types of link antibodies available. Monospecific link antibodies recognize only antibodies raised in a single species (mouse vs. rabbit, etc.), and these are the most economical. A number of manufacturers also market "multi-link" reagents that will bind to primary antibodies raised in multiple species. The latter are more convenient but are somewhat more expensive, although they perform just as well as monospecific reagents. As is the case with primary antibodies, the quality of link antibodies can also vary substantially, so do not be afraid to try different link antibodies from different manufacturers. We have found that link antibodies can be extremely stable, and in fact we have performed over 40,000 immunostains using one 1-liter batch of link antibody, without a detectable decrease in performance.

Detection Complex (ABC Reagent)

There are a wide variety of detection complexes available from different manufacturers, and many of them are quite good. I am partial to peroxidase-based detection systems, since in my experience they are about four times more sensitive than alkaline phosphatase-based detection systems. There may be differences in the degree of nonspecific background staining among detection reagents, with some of the newer streptavidin or neutravidin reagents showing cleaner backgrounds than the older avidin-based materials. The sensitivity of detection also varies somewhat from reagent to reagent. Some manufacturers market these reagents in a "ready to use" format, whereas others market these reagents in concentrated form, the latter in general being more economical. As mentioned above, if one is using a peroxidase-based detection complex in a batch method, it is critical that a peroxidase-stabilizing diluent be used to dilute the concentrated detection complex to its working dilution, or the detection complex is liable to "crash" within two weeks.

Development of Reaction Product (Incubation with Chromogen)

A number of different chromogens may be employed, depending on one's preferences. Diaminobenzidine (DAB, which gives a brown reaction product) is my personal favorite, since it gives a well localized and crisp end product that is easy to interpret, can be mounted in standard mounting media (since it is insoluble in organic solvents), and does not fade over time. Aminoethylcarbazole (AEC) results in a red reaction product preferred by some pathologists, although I detest this chromogen for a number of reasons. In my laboratory I have found AEC to be only about 1/4 as sensitive as DAB, and AEC must be mounted in aqueous-based mounting media, which gives this chromogen an annoying refractile fuzzy appearance on high power examination. It also has the tendency to deteriorate upon prolonged slide storage. AEC can be easy to interpret in "easy" cases, but in difficult diagnostic situations where one is trying to interpret reactivity of individual cells in a complex mixed cell population, I find DAB far preferable. There is at least one newer red-brown reaction product that can be mounted in standard mounting media (Vector Red), and we have evaluated this chromogen in our laboratory, although our results have been somewhat variable.

There are several options for performing the chromogen development step. In my prior laboratories, which were relatively low volume hospital laboratories, we performed this step by placing the slides on flat transparent plastic sheets, flooding the slides with copious amounts of chromogen, and watching the slides develop while viewing them at a microscope. When the technologist felt that the reaction had proceeded to the appropriate point, the slides were then placed in tap water to stop the reaction. One advantage of this method is that inadvertent mistakes in the staining procedure (for example, if a slide was placed in the wrong link antibody)

may be detected at this time (by failure of the positive control to stain), providing an opportunity to re-incubate the section and "rescue" the slide on the same day. In a large volume laboratory however, this is a bit too time-consuming, and for this reason we develop our slides in a batch mode by immersing them in large containers of DAB. After incubating the racks of slides for a predetermined amount of time, the racks are placed in tap water to stop the reaction. (The particular DAB that we employ is usable for several days in diluted format). As with all other reagents in IHC, all DAB is not the same, and I have found dramatically different staining results when employing DAB from different manufacturers.

Interestingly, we have also found that substantially improved results are obtained if the DAB is pre-warmed (in an incubation of oven) before use, and in our laboratory the optimal temperature is around 23-25°C. However, if the DAB gets too hot (around 35-40°C), there is an increase in nonspecific background.

Post-Chromogenic Enhancement

Another step that can noticeably improve the final result is post-chromogenic enhancement. This involves placing the slides in a solution that enhances the positivity with DAB. Currently we use copper sulfate for this purpose with good results, although in the past we used osmium tetroxide for a number of years (a fairly toxic chemical), and have also tried cobalt chloride, and nickel solutions. Our reagents and method are as follows:

Copper sulfate pentahydrate, 4% solution (25 ml vial), Sigma # C2284

To prepare, add one 25 ml vial to 175 ml of PBS (or 0.9% NaCl) in a staining dish. This will give you a 0.5% solution, which is the concentration used for post-chromogenic enhancement. The solution can be stored at room temperature for a month, and repeatedly re-used. After a month, discard the solution and prepare fresh material.

To perform enhancement, place the staining dish in a 25°C oven, and incubate the slides for 1-5 minutes in the copper sulfate solution.

Counterstaining

Believe it or not, the counterstain can have a big impact on the quality of the final result. As previously mentioned, we use a hematoxylin counterstain, but as any histologist knows, there are many different kinds of hematoxylin. Some of these have a gray-blue-brown color that often makes it appear that background is higher than it really is. However, the use of a Harris hematoxylin that is thoroughly rinsed in water or the use of a Gill's hematoxylin blued in ammonia water gives us a beautiful bright blue counterstain that contrasts very well with the DAB reaction product, and is very easy to mentally relate to the corresponding H&E section. As with all other reagents that we use, we have a favorite supplier of our hematoxylin counterstain, and the "same" hematoxylin from a different supplier has not necessarily given us equivalent results. (I have tried a number of counterstains other than hematoxylin, including light green, methylene blue, toluidine blue, and nuclear fast red, but I prefer hematoxylin.)

Azure B or Giemsa-Hematoxylin as a counterstain for pigmented melanocytic lesions

If one is dealing with heavily pigmented melanocytic lesions, it can at times be difficult to distinguish melanin pigment from DAB chromogen reaction product. In these types of case, the use of azure B as a counterstain (rather than hematoxylin) can be very useful (68-69, 69a.).

Azure B stains melanin a green-blue color, allowing it to be easily distinguished from the brown-black color of the DAB chromogen. Since Wright-Giemsa stains contain azure B, these reagents may also be employed for this purpose. We have developed a beautiful counterstain for heavily pigmented melanocytic lesions that involves a 5 minute stain in Wright-Giemsa stock solution, followed by a 20 second Hematoxylin counterstain (see appendix at end of handout). This gives excellent cytologic detail, has no effect on the color of DAB, and turns the melanin a blue-green color that is easily distinguished from DAB. This stain is also quite a bit more rapid than the Azure B staining procedures referenced above.

THE OPTIMAL MANUAL IHC STAINING METHOD

Now that we have discussed each step in the method in detail, I think it is useful to summarize the points discussed, and list what I have found in my laboratory to be the "optimal" manual IHC method. I should stress that this method may not be "optimal" for all labs, as there are certainly many different approaches to methodology in IHC. However, I have been constantly toying with IHC methodology since 1980, and the method listed below is the best one I have ever used.

IHC Method for Paraffin Sections – Streptavidin-Biotin-Peroxidase Method **ProPath Laboratory, Dallas, TX**

1. Cut and mount 3-5 micron paraffin sections toward one end of an adhesive slide, labeled with a solvent-resistant with the case number, antibody, and type of antigen retrieval. (Graphite pencils should not be used to label slides, since small microscopic particles of graphite may detach from the label area and lodge on top of the tissue during the staining procedure, resulting in microscopic collections of granular black material on top of the tissue). A multitumor tissue control slide should be mounted on the opposite end of the same slide. Remove batch reagents from the refrigerator so that they warm to room temperature before use.

2. Dry slides by placing them in 100-slide capacity plastic (microwave safe) racks in the microwave oven (1000 watt oven for 3 minutes on "high"). (Or, if you have the time, place them in a 56°C oven for one hour.)

3. Deparaffinize slides in xylene and graded alcohols to distilled water. (If slides have been fixed in B-5 or other heavy metal-containing fixative, **DO NOT DEZENKERIZE AT THIS POINT**. Wait until the immunostain is completed, and dezenkerize right before counterstaining.) Place slides in distilled water. (If pressure cooking is to be used, during the deparaffinization procedure, place the steamer rack in the bottom of the pressure cooker, add water to the pressure cooker, and then place it on the hotplate with the cover off. Begin heating the water with the hotplate set on "high". Also, remove the HIER solutions from the refrigerator so that they begin to warm to room temperature, and place them in plastic Coplin jars as needed.)

4. Perform protease digestion or HIER in a pressure cooker, vegetable steamer, or microwave oven as needed, depending on the requirements for detection of the antigen of interest.

Protease Digestion: Incubate slides with the appropriate protease for the appropriate time and at the appropriate temperature for the antigen of interest. After protease digestion is completed, rinse the slides in distilled water (NOT buffer, for reasons cited below). Use of warm tap water rinses may aid in removing the protease solution.

Pressure Cooking: **MAKE CERTAIN THAT YOU UNDERSTAND HOW TO OPERATE THE PRESSURE COOKER SAFELY.** **If using a standard household pressure cooker:** Place the slides into loosely covered plastic Coplin jars filled with the appropriate HIER solution, and then place the Coplin jars into the pressure cooker, on top of the steamer rack. The water level in the pressure cooker should be near the "max" level after the Coplin jars are placed in the cooker. Place the cover on the pressure cooker and turn the hotplate on "high". Begin timing the pressure cooker when the top pressure vent cover on the pressure cooker begins to rock back and forth, indicating full pressure is reached (about 15 p.s.i.). After full pressure is reached, the heat level on the hotplate should be reduced to maintain a steady slow rocking back and forth of the vent cover. After the appropriate cooking time (9 minutes in our lab), remove the pressure cooker from the hotplate, and allow it to cool for a specified "cool-down" time (25 minutes in our lab). Before opening the pressure cooker, slowly vent the pressure until all pressure is released. Then open the pressure cooker and remove the Coplin jars. Pour the antigen retrieval solution off, and then add warm tap water to the Coplin jars (to gradually cool slides), pour off this water, then add cool tap water to the Coplin jars to further gradually cool the slides. Now remove the slides and place them in distilled water (DO NOT PLACE THEM IN BUFFER AT THIS POINT, since the salts in the buffer solution may precipitate proteins in the egg white solution used in the step that follows). **If using a commercial electric pressure cooker with a timer** (BioCare Medical, Walnut Creek, CA): fill the pressure cooker with water to the appropriate level. Place the slides in Coplin jars (filled with the appropriate epitope retrieval solution) in the pressure cooker, close the lid, and turn the dial past 20 minutes and back to 5 minutes (This will then heat the slides at full pressure for 5 minutes). Simultaneously, set a separate timer for 48 minutes. When the 48-minute timer goes off, remove the slides from the pressure cooker and place them in distilled water. (DO NOT PLACE THEM IN BUFFER AT THIS POINT, since the salts in the buffer solution may precipitate proteins in the egg white solution used in the step that follows).

Vegetable Steamer HIER: Place sufficient water in the bottom of the steamer. Place slides in Coplin jars in the appropriate epitope retrieval solution, and place the Coplin jars in the steamer. Set the steamer for the desired time (we use 25 minutes). After the timer rings, remove the Coplin jars and let them cool down for 25 minutes. Place the slides in distilled water (DO NOT PLACE THEM IN BUFFER AT THIS POINT, since the salts in the buffer solution may precipitate proteins in the egg white solution used in the step that follows).

Microwave Oven Heating: If using a microwave oven for heating, always keep the same number of filled Coplin jars in the oven, regardless of how many slides are being processed (this will aid in consistent and reproducible heating). Heat the slides until the solution has boiled away down to the level of the bottom of the slide label (3-5 minutes in our lab), then refill the Coplin jars with HIER solution and repeat heating as needed (depending on requirements of the antigen of interest). Most antigens will require several 3-5 minute incubations, with refilling of the Coplin jars between heating periods. After heating is completed, pour the antigen retrieval solution off, and then add warm tap water to the Coplin jars (to gradually cool slides), pour off this water, then add cool tap water to the Coplin jars to further gradually cool the slides. Now remove the slides and place them in distilled water (DO NOT PLACE THEM IN BUFFER AT THIS POINT, since the salts in the buffer solution may precipitate proteins in the egg white solution used in the step that follows).

5. To block endogenous biotin activity, immerse slides (previously rinsed or bathed in distilled water) in dilute egg white solution (8 egg whites diluted with distilled water up to a final volume of 800 ml) at room temperature for 15 minutes. Now rinse the slides thoroughly with tap water from a squirt bottle. Several rinses are required to remove the somewhat tenacious egg white solution from the slides and slide rack. Next, immerse slides in 0.2% biotin in PBS for 15-

20 minutes at RT. (Alternatively, the slides may be immersed in a solution of 5% reconstituted dried milk, prepared by adding 25 grams of dried milk powder to 500 ml of PBS/Tween buffer). Rinse slides in tap water and then place them in PBS/Tween buffer rinsing solution. (NOTE: The dilute egg white solution can be stored in the refrigerator, and may be repeatedly used for one week). The biotin solution may be reused for several weeks to a month (possibly longer, although we have routinely changed it on a monthly basis). If using reconstituted dried milk as a source of biotin, we recommend preparing a fresh solution on a weekly basis (or whenever it starts to stink).

6. Incubate with primary antibody as follows:

Batch Manual Method: Blot excess PBS/Tween from the edges of the tissue sections, leaving just enough fluid to keep the sections moist. Place the slide in the appropriate plastic slide mailing container filled with appropriate dilution of desired primary antibody (the 5-slide capacity mailers hold about 22-25 ml), and place the containers vertically in a rack. Incubate at 25°C for 30 minutes with gentle orbital rotation (we set the rotator at 40 rpm). (Make certain that the reagents have reached room temperature before use). After the incubation is complete, remove slides from the plastic containers, and rinse with PBS/Tween from a squirt bottle. Transfer the slides into staining racks (20-25 slides per rack, or larger racks if available). Rinse the racks of slides in two changes of fresh DW/Tween rinsing solution (placed in two additional separate staining rack containers), 10-20 dips each. Drain the rinsing solution from the racks by tilting and then blotting on a paper towel.

Recipe for DW/Tween rinsing solution (for 10 liters): Add 2 ml Tween 20 to 10 liters of distilled water. Store at room temperature, good for at least one week

“Standard” Manual Method: Blot excess DW/Tween from the edges of the tissue sections, leaving just enough fluid to keep the sections moist. Add appropriately diluted primary antibody to cover the sections, and incubate with primary antibody at 25°C for 30 minutes with gentle orbital rotation of the slide tray (we set the rotator at 40 rpm). **Make certain the slide tray is not warped, and make sure that the slide tray and the rotator are level.** (Check with a carpenter's level periodically). Remove slides from the slide trays, and rinse with DW/Tween from a squirt bottle.

7. Incubate with biotinylated link antibody as follows:

Batch Manual Method: The staining rack container filled with the appropriate dilution of link antibody should be allowed to reach room temperature before using. Place the staining rack into the container of link antibody, and incubate at 25°C oven for 10 minutes with gentle orbital rotation as before. Rinse the racks of slides in three changes of fresh DW/Tween (placed in three additional separate staining rack containers), 10-20 dips each. Drain the DW/Tween from the racks by tilting and then blotting the racks on a paper towel.

“Standard” Manual Method: Blot excess DW/Tween from the edges of the tissue sections, leaving just enough fluid to keep the sections moist. Add appropriately diluted link antibody to cover the sections, and incubate with link antibody at 25°C for 10 minutes with gentle orbital rotation of the slide tray as before. Rinse slides with DW/Tween from a squirt bottle as before.

8. Quench endogenous peroxidase activity by placing the slides in 0.3% H₂O₂ in PBS with 0.1% sodium azide for 10-13 minutes at room temperature. The PBS with 0.1% sodium azide can be pre-made and stored in the refrigerator, but the H₂O₂ must be added immediately before use.

For best results, the quenching solution should be allowed to reach room temperature before use. Rinse with DW/Tween from a squirt bottle. If using the "batch staining" method, rinse in three changes of fresh DW/Tween as before.

9. Incubate with peroxidase-labeled streptavidin detection complex:

Batch Manual Method: Place the staining rack into the container of detection complex, and incubate at 25°C oven for 10 minutes with gentle orbital rotation as before. Rinse the rack of slides in three changes of fresh DW/Tween, (placed in three additional separate staining rack containers), 10-20 dips each. Drain the DW/Tween rinsing solution from the racks by tilting and then blotting the racks on a paper towel.

“Standard” Manual Method: Blot excess DW/Tween from the edges of the tissue sections, leaving just enough fluid to keep the sections moist. Add appropriately prepared detection complex to cover the sections, and incubate at 25°C for 10 minutes with gentle orbital rotation of the slide tray as before. Rinse slides with DW/Tween from a squirt bottle as before.

10. Develop reaction product by immersing the slides in DAB solution that is pre-warmed to 23-25°C (the solution may be warmed in an incubation oven). Incubate for the appropriate time (about 4-5 minutes in our lab), and then stop reaction by placing the slides into tap water. Alternatively, for smaller numbers of slides, development can be accomplished by placing the slides on transparent sheets of plexiglass, and flooding the slides with COPIOUS amounts of DAB. Monitor the reaction under a microscope, and stop reaction when appropriate by placing the slides in tap water

11. Remove slides from water, and perform **chromogenic enhancement** by placing the slides in 0.5% Copper Sulfate in PBS with Tween (or normal saline) for 1-5 minutes at 25°C with gentle orbital rotation. Rinse in distilled water. (The copper sulfate solution can be reused for 1 month.)

12. If needed, slides may be "**De-Zenkerized**" at this point. (alcoholic iodine solution for 5 minutes, rinse well in tap water, then 5% sodium thiosulfate for 5 minutes, then rinse well in tap water).

13. Counterstain slides in hematoxylin, rinse thoroughly in water, dehydrate, and coverslip.

USE OF POLYMER-BASED DETECTION SYSTEMS

In the past several years, several detection systems employing link antibodies and marker enzymes attached to a polymer backbone have been described, including EnVision marketed by Dako (Carpinteria, CA) (83), and PowerVision marketed by ImmunoVision Technologies (Daly City, CA) (84). These detection systems do not rely on the avidin-biotin interaction for localization of the marker enzyme to the antigen. Therefore, it is not necessary to take steps to block endogenous biotin activity. In addition, since these methods combine the link antibody step and the detection complex incubation step into a single step, it is a more rapid method. These detection systems perform very well, and they indeed do have distinct advantages. Their main disadvantage is that they are more costly than standard avidin-biotin reagents. Nevertheless, depending on particular laboratories needs, these reagents may be worth the added expense. In our own particular situation at ProPath, we have been using one of these reagents (PowerVision) in a batch mode for some time, and we have been extremely pleased with the results.

TYRAMINE ENHANCEMENT

At this point, I will briefly discuss the use of a sensitivity enhancing IHC technique employing biotinylated tyramine that has been reported by a number of investigators. To my knowledge the use of this technique was first described in immunoassays by Bobrow and associates in 1989 (70), and has been subsequently applied to immunohistochemical stains by others (71-73). Various groups or vendors have also referred to this as “catalyzed reporter deposition”, “catalyzed signal amplification” or “ImmunoMax”.

This amplification method takes advantage of the ability of peroxidase enzyme to catalyze the deposition of large amounts of biotinylated tyramine in the immediate area (and for this reason a peroxidase-based detection system must be used). The primary antibody, link antibody, and peroxidase-conjugated avidin (or streptavidin) detection complex incubations are performed as usual. At this point, the slides are incubated with biotinylated tyramine. The peroxidase enzyme (from the detection complex) catalyzes the deposition of large amounts of biotinylated tyramine in the immediate area. This has the effect of dramatically increasing the number of biotin molecules in the region of the detection complex. Another incubation with peroxidase-conjugated avidin detection complex follows, and additional detection complex will bind to the free biotin that has been deposited in the area during the addition of biotinylated tyramine. The end result is amplification of the number of peroxidase molecules that are localized to the area of the antigen of interest. Finally, the chromogen incubation is performed as usual, and because there are more peroxidase molecules attached to the antigen, the detection signal is stronger than with the routine (non-tyramine based) method.

Tyramine enhancement can dramatically increase the sensitivity of detection of a variety of antigens (anywhere from 10 to 200 fold), and it can allow the detection of antigens that otherwise may not be detectable. However, there are also significant problems that may need to be overcome when employing these procedures. Background staining can be a severe problem, and can render the technique essentially useless in some circumstances. Because the degree of amplification can be very great, any endogenous biotin in the tissue (that becomes bound by detection complex) will also be greatly amplified, leading to increased background. Any endogenous peroxidase in the specimen (that has not been absolutely completely quenched) will also be amplified greatly, which can be particularly problematic on bone marrow or other specimens that contain a lot of blood (like cell blocks of bloody FNA's or bloody fluids). Another problem that we have experienced is a bothersome “reagent trapping” artifact that occurs when small microscopic pieces of tissue lift away from the slide, a particular problem in tissues that have dense collagen.

We have worked with this technique in our laboratory and use it for a small number of antibodies, but it took a great deal of time and effort working with the technique to get it to the point that we could use it for diagnostic purposes. Both the time of the tyramine incubation and the concentration of the tyramine used must be monitored, as increasing either of these variables results in increased sensitivity, which if too great will lead to significant background problems. I still avoid working with tyramine unless I have no other choice, as in my experience it is a very “high maintenance” procedure that often requires “tweaking” on nearly a daily basis. Indeed it is very powerful, but I liken the technique to providing a person with a loaded gun: if that person is not cautious and doesn't really know how to handle it, bad things are going to happen. I would encourage anyone to try this technique if they have the inclination and the time, but I would be remiss if I said that the technique is not without its own problems and frustrations. (Parenthetically, if any reader uses this technique routinely on a wide range of diagnostic specimens with consistent and fabulous results, please call me and tell me what I'm doing wrong!)

POST ANALYTIC (POST-STAINING) VARIABLES

Basically, post-staining variables rest in the brain of the person interpreting the stains, and no degree of technical excellence can control for these highly variable factors. As previously discussed, it is important for the technologist and the pathologist to be able to recognize the features of a true positive and a false positive stain. Also, if these stains are to be used to best advantage, those interpreting the stains need to be well versed in the expected patterns of reactivity with the markers being employed and the tissues being studied. Knowing the names of the antibodies is not enough, as they can be very misleading. For example, it is not uncommon for some types of lymphomas to stain with epithelial membrane antigen, and so-called neuron-specific enolase (NSE) antibodies are far from being neuron-specific. (Editorial comment: In my opinion, anyone who uses a positive NSE stain by itself as an indicator of neuroendocrine differentiation is crazy, and it is obvious that such pathologists have never used multitumor sandwich blocks as positive control specimens, or they would immediately realize the error of their ways).

The importance of a **PANEL** approach to IHC should also be mentioned here. It is crucial that cases be approached with a differential diagnosis (i.e., list of likely possible diagnoses) in mind, that is developed after careful study of the H&E sections in conjunction with clinical history, and that appropriate panels of antibodies be selected to narrow this differential diagnosis. Since "**We see only what we know**", it is important for the pathologist to be a good morphologist before he or she can become a good immunohistochemist. If the list of differential diagnostic possibilities does not include the correct diagnosis, it may never be rendered, regardless of the number of immunostains performed on the case.

Immunohistochemical Guilt

Some pathologists suffer from intense guilt every time they order an immunostain, and their level of guilt rises (sometimes exponentially) with each immunostain that is ordered. This guilt is obvious when I get phone calls asking me questions such as "What two or three stains do I need to order to diagnose a small blue cell tumor in a child?" I have seen many cases where this guilt has directly contributed to a misdiagnosis, since the pathologist's overwhelming concern for running up the bill led to insufficient and incomplete analysis of the case, resulting in diagnostic errors. My advice to these pathologists is to get over it (the "tough love" approach), or see a therapist if needed, as the cost of an erroneous diagnosis is far greater than the cost of an appropriate panel of immunostains. In the grand scheme of things, a large battery of immunostains costing \$1000-2000 may be a very wise investment in overall patient care, particularly when one considers the costs involved in re-biopsy, or the costs involved in multiple diagnostic procedures. (For example, the cost of an uncomplicated outpatient screening colonoscopy procedure in Dallas in 2001 is about \$3000-3500).

QUALITY CONTROL OF DIAGNOSTIC IHC

The Role of Multitumor Tissue Blocks

Poor quality control has been the downfall of many diagnostic IHC laboratories. There is absolutely no question in my mind that **the key to effective and efficient quality control is the extensive use of multitumor control slides for quality control purposes**. These preparations were the brainchild of Dr. Hector Battifora, certainly one of the "heavy hitters" of IHC, and he actually has a patent on his idea. I prepare my own multitumor blocks (usually containing

between 60 and 80 different tumors) from routine diagnostic surgical pathology material received in the hospital and outpatient laboratories affiliated with ProPath. I have developed modifications of Dr. Battifora's methods that allow the placement of numerous different small pieces of tumor in a well-organized compact grid, which takes up only a minority of the surface of a microscopic slide (74). My method can be used in any histology laboratory, since it does not require any special equipment. I have continued to modify and simplify the method of preparation, and I will illustrate the most recent modifications during the presentation (as well as at the end of the handout). There are also a variety of other published methods for similar preparations, but I believe the method I use is best for preparing multitumor sections in large numbers. The method of preparation is not that crucial, and I would suggest that labs use whichever method they feel best suits their needs. However, I do believe it is crucial that every diagnostic IHC laboratory use these types of preparations.

Multitumor sandwich blocks (MSB's) have many uses in quality control. Their use in assessment of optimal titers of primary antibodies has already been addressed. They also provide a rapid and efficient way to screen unusual or new antibodies for sensitivity and specificity, and to compare them with current similar reagents. Finally, they provide an ideal preparation for use as positive control sections. If the component tumors in an MSB are chosen appropriately, a single MSB can be used as a positive control section for essentially any stain that is desired, obviating the need for cutting a different positive control section for every different antibody stain that is ordered. (Obviously, histotechnologists appreciate this greatly). These preparations are compact, so large numbers can be pre-cut and mounted toward one end of the microscopic slides, and stored for use as positive control sections. When a case is to be stained, the case tissue is simply mounted on the other end of the microscopic slide. Therefore, the case tissue and the MSB positive control tissue section are mounted on the same slide, which can nearly halve the number of slides that need to be manipulated by the technologists. By mounting the MSB positive control section and the case tissue on the same slide, we are assured that both tissue sections are handled in an identical fashion, which may not occur if the positive control section and the case tissue are mounted on different slides. If there is ever any question about the performance of a particular stain on a case, one simply has to view the MSB section (mounted on the same slide) to quickly assess the sensitivity and the specificity of the stain. In my opinion, this arrangement provides a level of continuous ongoing quality control and continuous ongoing documentation of that quality that is unbeatable.

The Use of Vimentin in Quality Control

The use of vimentin stains as a quality control measure has been described by Battifora (75). He recommends performing a vimentin stain on every case, using an antibody to a formalin-sensitive epitope, to assess the degree of antigen damage in tissue fixed in formalin. Vimentin is an excellent choice for this purpose, since there are always some vimentin-containing cells in nearly any tissue taken for biopsy purposes. There are large amounts of vimentin in endothelial cells that line blood vessels, as well as in mesenchymal stromal cells. If the vimentin stain shows very weak staining on a case or in a particular area on the slide, it is probable that these areas have been overfixed in formalin and are showing the effects of antigenic damage. As such, one needs to keep in mind the degree of antigen damage when assessing the results of the other stains. In a case that shows much antigen damage on the vimentin stain, very weak positivity of the cells in question may be a very significant positive finding, in contrast to other cases (that do not show antigen damage) where one might be tempted to ignore a weakly positive or trace positive stain. As mentioned previously, the use of protease digestion procedures or HIER techniques are essential for optimal staining of antigenically damaged tissue.

Control Slides: How many and what kind do you need?

From time to time I am asked to review immunostains performed in other laboratories, and this has made me realize that there is some degree of confusion regarding the number of needed control slides. I believe that much of this confusion is related to the College of American Pathologists Accreditation Checklist for Immunohistochemistry Laboratories, which in my opinion has vague and confusing language in their requirements for necessary controls. On more than one occasion I have seen cases that include a negative control slide for each antigen being stained, even if only one tissue block is used. For example, a block that has CK, EMA, LCA, S100, and HMB-45 stains will also have five separate negative control slides. This is unnecessary, and is a waste of time and reagents. My approach to control slides is as follows:

Negative Control Slide(s): If you are using only one type of epitope retrieval for all slides, only one negative control slide needs to be run for every different tissue block that is stained. For example, if ten stains (all heated in citrate buffer for antigen retrieval) are run on a particular tissue block, you need to only run one negative control slide (NOT ten!!) The negative control slide should consist of a section of the tissue block being studied, and it should be treated identically to all other slides, with the exception that the primary antibody is omitted and replaced with some other solution (tissue culture media, buffer, dilute non-immune serum, etc.). **It is particularly important that the negative control slide be subjected to the same epitope retrieval procedures** (protease, microwave, pressure cooker, etc.) as the primary antibody, since these epitope retrieval procedures can markedly intensify endogenous biotin artifacts. These artifacts can be very prominent, and are easily misinterpreted as true reactivity by the unwary. They are seen most often in cells that have abundant mitochondria, such as kidney, liver, and oncocytomas. If a particular run of stains includes some slides treated with protease and others subjected to epitope retrieval, there should be one negative control slide run for each different type of protease or epitope retrieval technique used. For example, if some slides are heated in dilute urea and all others in citrate buffer, you need one negative control slide placed in citrate buffer, and another negative control slide placed in the dilute urea. In place of primary antibody, I recommend using McCoy's medium for the negative control slide, which is a tissue culture medium that is similar to supernatants from monoclonal antibody preparations minus the antibody. Other authors suggest that dilute normal mouse ascites fluid (for monoclonal antibodies) or dilute normal rabbit serum (for polyclonal antibodies) be used in place of primary antibody for the negative control sections, but after using those in my first lab and comparing them to what we use now, I can say that I believe that McCoy's medium works better from a negative control standpoint.

Positive Control Slides: A positive control section needs to be run with every different antibody that is used. The positive control section should be mounted on the same slide as the tissue section being stained, as that is the only way to assure that the case tissue and the control tissue are handled in the same fashion. From my comments above, you can guess that I believe that multitissue sandwich block preparations (or a similar type of multitumor block preparation) should be used as positive control sections. These preparations allow you to assess multiple known positive tissues and also multiple known negative tissues (since it is just as important to document that known negative cases are indeed negative). In many laboratories, the positive control sections consist of a single piece of tissue from a case known to be positive for the antigen in question. In my opinion, this is grossly inadequate for a positive control and can be outright dangerous, since this does not evaluate and document the sensitivity (how many true positive tissues react positively) or the specificity (how many true negative tissues fail to react with the antibody). If at all possible, these two factors should be documented with every stain that is

performed, and this is easily done by using multitumor sandwich block preparations or some other similar multitumor block preparation.

WORKING UP A NEW ANTIBODY

Working up a new antibody is a task that confronts all IHC laboratories from time to time. As previously mentioned, you can never believe a manufacturer's claims (either for titer, sensitivity, or specificity) before testing the antibody in your own lab. (Heaven forbid if you purchase a "predilute ready-to-use antibody" without any further testing. This is one of the mortal sins of IHC.) Additionally, don't necessarily believe the manufacturer's recommendations for proteases or epitope retrieval procedures. They may be satisfactory, but you may find your own method that works much better. Two of the first questions that need to be answered in this situation are as follows:

1. Does the antibody require any type of epitope retrieval, and if so, what is the best type of epitope retrieval (protease, pressure cooker, vegetable steamer, microwave oven), and what epitope retrieval solution or protease is best?
2. What is the optimal titer of the antibody for the conditions and methodology in my lab?

The answers to these questions can be sought in several ways, although I think it is usually best to try to answer the first question before determining the optimal titer, since the optimal titer will vary depending upon what type of epitope retrieval is employed. As such, when I receive a new antibody in the lab, my approach to working it up is as follows:

1. Mount tissue sections from an expected positive case on the same slides as the multitumor tissue block control sections. These slides will be used for all steps in the workup of the antibody. (In this fashion, you will be evaluating a number of expected negative tumors and frequently also a number of different positive tumors of varying antigen densities, using only 1 slide.)

2. Dilute the primary antibody to the titer suggested by the manufacturer. (It may be way off, but you have to start somewhere). Run the first set of slides as follows:

- a) one slide with no epitope retrieval
- b) one or more slides with protease(s) of choice (we use ficin, pepsin, and occasionally other proteases)
- c) several slides in different heat-induced epitope retrieval solutions, (citrate buffer, Tris buffer, EDTA, urea, etc.), using either a pressure cooker (preferred), vegetable steamer, or a microwave oven

At this point, examine the slides and see if you can get an idea of whether or not epitope retrieval is needed, and if so, which method is most effective. If everything is staining strongly or it seems like there is horrific background staining on everything, your primary antibody titer is probably too concentrated, and at this point you should repeat the steps above using a more dilute primary antibody titer. If there is no staining at all or very weak staining, repeat the steps above with a more concentrated primary antibody titer.

Using the optimal method of antigen retrieval identified in the steps above, try a series of stains using 4 to 6 two-fold serial dilutions of the antibody. For example, if you are starting at a

primary antibody titer of 1:100, try additional stains with the primary antibody diluted at 1:200, 1:400, 1:800, 1:1600, and 1:3200. Select the best titer after studying the sections, with a particular eye to sensitivity and specificity. These factors can be judged by keeping a detailed record of the contents and location of the individual tumors present in your multitumor tissue control block. Some additional fine-tuning of the titer may be required (using either more concentrated or more dilute primary antibody preparations) but this procedure usually works quite well and generally only takes two days (or only one day if your lab does 2 runs daily.)

CRYOSTAT SECTIONS

In many laboratories, paraffin section IHC is the dominant type of IHC performed, so experience in dealing with cryostat sections may be lacking. In my initial forays into cryostat section IHC, I was frustrated by poor stains that had terrible morphology, but I realize now that much of my frustration was from following published methods that advocated air-drying of cryostat sections before immunostaining. Studies in my prior laboratory showed that this was a big mistake, and far superior results are obtained if the sections are not allowed to air dry at any time during the staining procedure (76).

Optimizing Cryostat Morphology

The key points to remember in dealing with cryostat section IHC are **Rapid Freezing** of the specimen and **Rapid Fixation (Without Any Air-Drying)**. Slow freezing allows ice crystals to form in the tissues during the freezing process, and this destroys the cells and dramatically distorts the tissue. Flash freezing (in liquid nitrogen or isopentane) is best, although good results can be obtained without these if care is taken to freeze the tissue as fast as possible. The tissue should be placed on a cryostat chuck on which a frozen flat surface of Optimal Cutting Temperature (OCT) media has been prepared. Additional OCT is then added to cover the specimen, and the heat extractor of the cryostat is quickly placed on the top of the chuck. This allows for rapid freezing with a minimum of ice crystal formation in the tissue. In order to get the best sections possible, in my opinion the sections should be cut as soon as possible after the tissue has first been frozen. Although flash freezing in liquid nitrogen prevents ice crystal formation, any lapse in meticulous handling (i.e. warming of all or part of the tissue by warm fingers, warm container, etc) during removal and recutting of stored frozen tissue can lead to bad artifacts (from parts of the tissue thawing and slowly re-freezing). If you are dealing with flash frozen tissue, it is best to put the tissue into the cryostat and allow it to reach cryostat temperature (-18 to -20 degrees C), before taking your cryostat sections, since shattering of tissue sections may occur if the tissue is too cold when being cut.

Fixation of Cryostat Sections

As mentioned, the sections should be rapidly fixed after thaw-mounting the sections on the slides without any air-drying. Fixation for a minute or so seems to be sufficient, and we have not found fixation time to be a critical factor for dealing with most antigens.

The optimal cryostat section fixative depends on the antigens being stained. For many antigens, 95% alcohol works satisfactorily. **Buffered Formalin-Acetone Fixative**, pH 6.6, is the preferred fixative for estrogen and progesterone receptors, TDT, and B and T cell antigens. This fixative is prepared by mixing 150 mg Na₂HPO₄, 1200 mg KH₂PO₄, 300 ml water, 450 ml acetone, and 250 ml of 37-40% formaldehyde. It can be stored in the refrigerator for up to one month.

After fixing the slides, they should be transferred to PBS, and can be stored in PBS in the refrigerator until they can be stained. We have found that B and T cell antigens survive well at least for 5 days in the refrigerator, and other antigens (cytokeratin, etc.) survive for months. Morphology is also well preserved for months (up to 16 months, provided the PBS doesn't get contaminated by bacterial growth).

Advantages of Cryostat Section IHC

Since cryostat section slides for IHC may not require deparaffinization, protease digestion, or HIER methods (although they may improve staining, see below), they can be useful for obtaining a very rapid diagnosis on a difficult case seen at frozen section, and can allow a definitive diagnosis to be made even before the H&E paraffin sections are examined. Cryostat section IHC is also satisfactory for assessing estrogen and progesterone receptor status on breast cancers that are found at frozen section, although this is now largely unnecessary because of the excellent performance of estrogen and progesterone receptor immunostains in paraffin sections.

Disadvantages of Cryostat Section IHC

Although morphology of cryostat section IHC can be very good, the paraffin section stains always seem to be better and easier to interpret. Also, calcification or high fat content of a tissue specimen may make obtaining good cryostat sections difficult, so IHC on these types of specimens may not be the greatest.

Epitope Retrieval for Cryostat Sections

From a theoretical standpoint, one would think that epitope retrieval for cryostat sections would not be necessary, since the antigens in most cases will not have been exposed to the deleterious effects of formalin fixation. At the time of this writing (August 2001), I have not systematically evaluated the effects of epitope retrieval on cryostat sections, mainly because I now run a referral lab that does not handle frozen material. However, I have recently discovered that the results of IHC staining on cytologic smears (both air-dried and alcohol fixed) can be improved (sometimes dramatically) for many antigens by using epitope retrieval (see section below on cytologic specimens), and I have no reason to doubt that cryostat sections would be any different. I would encourage every lab to evaluate the possible beneficial effects of epitope retrieval, since I suspect that these procedures may very well enhance the reactivity of cryostat sections (as well as paraffin sections and cytologic smears).

CYTOLOGIC SPECIMENS

General Comments on IHC and Cytologic Specimens

I think the best advice that can be given when thinking about doing IHC on cytologic specimens is the following: **GET A CELL BLOCK!!**. The reason for giving this advice is that performing IHC on cell block preparations is the same as dealing with any other paraffin section. Additionally, if you have a good cell block, you can perform many stains on the material, whereas if you only have smears, theoretically you need one smear for a negative control slide, and one additional smear for every antigen that you want to stain (unless you can employ the "cell transfer" techniques discussed below). As you all know, in many cases (especially

challenging fine needle aspiration or FNA) cases, you may not have as many smears as you need. One trick that we have used extensively to help us get cell block material is the use of thrombin and outdated plasma (obtained from the blood bank) as "gelling" agents. The thrombin and plasma are placed in convenient dropper bottles. Even if there is only a small drop of blood or other fluid on a slide (e.g., from a FNA specimen), adding a drop of thrombin (or a drop of thrombin followed by a drop of plasma) will cause the material to gel, and it can be scraped off the slide (using a single-edged razor blade or the edge of another slide) and placed in formalin, where it will remain in a compact cohesive form that is easy to process as a cell block into paraffin. Anyone who has ever tried to fix a drop of blood or other fluid in formalin without further manipulations knows that the drop of blood or fluid will immediately disperse into unusable slurry. The point of this discussion is this: **IT IS FAR BETTER TO HAVE ONE DIAGNOSTIC CYTOLOGIC SLIDE WITH AN ACCOMPANYING CELL BLOCK, THAN HAVING MULTIPLE DIAGNOSTIC CYTOLOGIC SLIDES (all showing the same thing!) WITHOUT AN ACCOMPANYING CELL BLOCK.** I think that in many FNA cases, much good diagnostic material that could be used for cell block is wasted by smearing it out on additional slides, and often efforts at obtaining a cell block are only an afterthought. **In the grand scheme of things, I know from personal experience that cytopathologist educators would serve the interests of patient care far better if they spent far more time teaching pathologists how to obtain adequate cell blocks,** in addition to the time that they spend teaching the fine points of morphologic cytopathologic diagnosis. I have seen countless cases in my practice where multiple highly-trained subspecialty board-certified cytopathologists end up scratching their hands on difficult cases, and the lack of cell block material in these cases has been a major problem (despite the presence of innumerable beautifully-prepared cytologic smears). Fortunately, we are now able to address these types of problem cases in a far more effective fashion (discussed below under "*Staining of Previously Stained Slides*").

IHC on Cytologic Smears or Slides (or, Making the Best of a Bad Situation)

Having made the above comments about the importance of obtaining material for cell block, I certainly realize that there are cases where IHC is very useful and a cell block is not available. The optimal type of smear varies depending on the type of case. If dealing with a lymphoma, leukemia, or a CSF specimen that is potentially involved by a lymphohematopoietic process, the smears or cytocentrifuge specimens are probably best air-dried and stained in a fashion identical to that used for blood and bone marrow smears. Alcohol-fixed material will also work for lymphomas and leukemias, although the cytologic features of the cells may be more difficult to appreciate for hematopathologists who prefer air-dried material. For other types of cases, immediate fixation of the smears (**without any air-drying**) is recommended, and the suggestions for fixatives are basically the same as for cryostat fixatives. For estrogen and progesterone receptor (ER and PR) stains, the smears should be immediately fixed in cold (4°C) buffered formalin-acetone fixative, pH 6.6, although 95% ethanol works satisfactorily. It is important to realize that any air-drying of smears may make reactivity to ER and PR more difficult to detect. For most other types of non-lymphoma / leukemia cases, immediate fixation in 95% alcohol is suggested, providing you incorporate a step for blocking of endogenous biotin activity, as occasionally endogenous biotin results in bothersome artifacts in cytologic preparations that can interfere with interpretation of the results.

The Use of ThinPrep Specimens for Cytologic IHC

Leung et al (80) evaluated the use of ThinPrep smears for IHC on 70 specimens, including 56 FNA is, 13 body fluids, and 1 year and specimen. They were processed on the ThinPrep processor, fixed in ethanol, spray fixed upon removal, and stored at -70 °C until processed. A

standard avidin biotin peroxidase method was used, but no antigen retrieval was used and there was no step for blocking of endogenous biotin. Satisfactory results were obtained in all cases except 9 cases of lymphoma, that reportedly all showed co-expression of kappa, lambda, CD20 (L26), and CD45RO (UCHL-1). These authors noted that the ThinPrep process lyses red blood cells and washes away proteinaceous debris, giving a clean background that leads to specimens that are easier to interpret.

Storage of FNA Cytospins Before IHC Staining

The use of several preservative solutions for cytokeratin AE1/AE3 immunostains was reported by Kaplan et al (81), who found that storage of slides in CytoLyt, PreservCyt, or 50% ethanol for up to five days yielded satisfactory preservation of cytokeratin antigen, although specimens preserved in CytoLyt tended to show the best morphology. They obtained optimal results with subsequent fixation for 5 minutes in either 95% ethanol or acetone.

Technical Problems in Cytologic IHC: Recommendations of Leong et al

A nice review of technical problems associated with cytologic IHC was published in 1999 by Leong and colleagues (82). These authors also stressed the importance of obtaining a cell block whenever possible. They advocate rehydration of air-dried smears with normal saline, since this lyses RBC's and tends to reduce background staining. Air-drying of smears is favored by these authors (something I would avoid), since in their experience this led to better cell adherence. They also advocate storing slides at room temperature for up to 24 hours before fixation. In their experience, the optimal overall fixative is 0.1% formal saline (0.25 ml 40% formalin in 100 ml normal saline), and they recommend fixing specimens for 2-14 hours, followed by post fixation in 95-100% ethanol for 10 minutes. For ER, PR, Ki-67, and HER-2 immunostains, they advocate air-drying the smears for 20 minutes-14 hours at room temperature, followed by 2-4 hours of fixation in 10% neutral buffered formalin (although 0.1% formal saline also worked as well as a fixative for this purpose). These recommendations are very helpful, although they require that those performing the immunostains have complete control over processing of the specimens, a luxury which does not apply in a referral setting such as my lab, (where we are forced to deal with whenever is sent to us). Therefore, in my particular setting, I have not been able to evaluate many of these recommendations. In addition, considering our desire and the client's desire for rapid results, long pre-staining storage times and long fixation times are to be avoided if suitable alternatives can be found.

Epitope Retrieval for Cytologic Specimens

Since cytologic smears are not routinely subjected to formalin, one could reasonably conclude that epitope retrieval techniques are unnecessary for IHC staining of these specimens. Indeed, that is what I thought until July 1998, when I began to study the effects of pressure cooker HIER on IHC staining of cytologic smears. Much to my surprise, staining for many antigens was actually improved when a step for pressure cooker HIER was included, particularly in air-dried smears, where the improvement in the stains was often quite dramatic. At the time of this writing, I have evaluated the effect of pressure cooker HIER on alcohol-fixed and air-dried cytologic smears for the following antibodies: cytokeratin AE1/AE3, cytokeratin low molecular weight (LMW) clone 5D3, cytokeratin 20, cytokeratin 7, neurofilament, synaptophysin, estrogen receptor (clone 1D5), progesterone receptor (clone 1294), and vimentin. In the case of air-dried smears, results were improved (often dramatically so) by including a step of pressure cooker HIER in the staining method (making the staining procedure essentially identical to the method used for paraffin sections). In the case of alcohol-fixed smears, only vimentin, cytokeratin 7, and

progesterone receptor (clone 1294) were better when the HIER step was omitted, although all antigens were still detectable. I encourage all immunohistochemists to try HIER on their cytologic specimens, since results can often be substantially improved by using HIER.

As with paraffin sections, one always needs to be mindful of whether or not adhesive slides have been used to prepare the cytologic smears that you will be using for immunostains. If they have been placed on adhesive slides, then HIER procedures can be undertaken without further manipulations of the material, and in general cell adhesion will not be a problem. However, if your cytologic smears have been placed on regular (non-adhesive) slides, I would NOT recommend trying HIER or protease procedures on them, as the diagnostic material will likely detach from the slides, and you will be left with no material to evaluate. If you are starting with non-adhesive slides, use the tissue or cell transfer techniques discussed below to transfer the material to adhesive slides, so that you can employ HIER procedures as needed.

Pitfalls in Interpretation of Cytologic IHC

When examining IHC stains of cytologic smears, one must be wary of interpreting three-dimensional clusters of cells, as in some cases there is a certain amount of reagent "trapping" that can occur, leading to spurious staining of three-dimensional clusters. This spurious staining lacks the normal cell-to-cell variability in intensity of staining, so attention to that staining pattern can be a helpful tip-off for recognition of the artifact. Another pitfall that we have encountered is spurious staining of cells that are phagocytosing other cells, particularly if the phagocytosed cells are neutrophils.

STAINING OF PREVIOUSLY (Pap, H&E, or Immuno-) STAINED SLIDES (Tissue Transfer IHC)

Often pathologists find themselves in the situation of trying to make a definitive diagnosis on minuscule fragments of tissue, and we have all experienced the vexing problem of running out of tissue in the paraffin block, or running out of smears on a difficult cytologic specimen. In this situation, it is useful to remember that immunohistochemical stains can be performed on previously stained slides. The first step is to have the histologists de-stain the slides and rehydrate them to water, and then place them in PBS. At this point the slides are ready for immunostaining. An important point to remember is that the majority of routine H&E sections or cytologic smears are not placed on adhesive slides, so any attempts at antigen retrieval (using proteases, vegetable steamers, microwaves, or pressure cooking) in this situation will cause the tissue or cells to fall off the slide. Therefore, **DO NOT ATTEMPT ANY HIER OR PROTEASE DIGESTION PROCEDURES IF YOU ARE STARTING WITH A SECTION OR SMEAR ON A NON-ADHESIVE SLIDE.** (If you are dealing with a situation that you know will require protease digestion or HIER for a good result, you are better off employing the "cell-transfer" or "peel and stick" technique discussed below in the next paragraph.) On many occasions, I have been successful in establishing a definitive diagnosis on small biopsies of de-stained H&E sections by simply doing the immunostains over the de-stained H&E, saving the patient the time and expense of a repeat biopsy or a more extensive surgical procedure. I have also performed immunostains on top of immunostains performed in other laboratories, where additional stains not initially performed were needed to establish a definitive diagnosis.

"Tissue Transfer" (or "Peel and Stick") Techniques for Immunostaining of Specimens

In 1994 Sherman et al described a method of cell transfer that can facilitate immunostaining of small cytologic specimens (78), when only one or two cytologic slides are available. This method is illustrated graphically in an addendum at the end of handout. First of all, the pathologist should review the slide(s) to be stained, and the different areas of the slide that are to be stained should be marked on the slide with a marking pen. These same areas should then be marked on the BACK SURFACE of the slide with a diamond-tipped pencil (so that the technologists will know which areas of the material to use for immunostaining, since the coverslip will be removed in the next step). After soaking off the coverslip, the slides are placed horizontally in an oven (we use a 56°C oven) and liquid coverglass medium is applied to the surface of the slide, to completely cover the areas to be immunostained. The medium is then allowed to harden in the oven (which may take several hours or more). (The liquid coverglass medium, called "Mount Quick", can be obtained from Newcomer Supply, 2217B Parview Road, Middleton, WI 53562, 608 831-7888, 800 383-7799, fax 608 931-0866. They can also provide a detailed procedure). After the coverglass medium has hardened, the slides are placed in warm water for an hour or so, and this allows the removal of the liquid coverglass medium along with the attached cells. The coverglass medium is then cut into smaller pieces with a razor blade, corresponding to the previous markings on the back surface of the slide. Each of these pieces is transferred to a different adhesive slide (keeping the surface that was in contact with the slide the same on the new slide), along with appropriate control material on the same slide if possible. The slides are then dried in an oven, and the liquid coverglass medium is then removed by soaking the slides in xylene. The slides are then rehydrated, and immunostains are performed on the slides as usual (including any epitope retrieval procedures that may be required for optimal staining). We have used this technique countless times in our lab, and it works extremely well for us. (In one case we were able to perform 30 immunostains on material from a single previously H&E stained FNA cytologic smear!) (Undoubtedly the immunohistochemical equivalent of "my dad can beat up your dad") In 1993 Mehta and Battifora (79) described a similar technique called the "peel and stick" method for use with paraffin sections, employing a liquid coverslip medium called "Harleco Krystalon" (EM Diagnostic Systems, Inc., Gibbstown, NJ). The "peel and stick" technique has minor differences but is similar in principal to the "cell transfer" technique described above. We have also used the "cell transfer" technique extensively on previously stained H&E tissue sections, so indeed it works just as well for histologic sections as cytologic smears. It is particularly useful on small needle biopsies of prostate, where the tiny suspicious areas frequently disappear on deeper sections, necessitating use of H&E levels for tissue transfer and subsequent immunostaining. (In a small number of cases however, the tissue transfer immunostains are unexpectedly weak on prostate biopsies. At this point I do not have the exact explanation for this relatively uncommon problem, although I suspect that these cases may have suffered from suboptimal handling before the H&E's were made).

In general, if pressure cooker HIER is employed in the immunostaining of previously-stained tissue or cell-transferred material, de-staining of the slides is unnecessary, as the pressure cooker HIER step destains H&E's, pap stains, and Wright-Giemsa stains nicely.

ProPath Approach to Immunostains on Previously-Stained Slides

If material is on **non-adhesive slides**: use tissue transfer immunohistochemistry as follows:

1. Examine slide, select areas to be immunostained, and mark them on the BACK surface of the slide with a diamond tipped pencil.
2. Remove coverslip, add liquid coverglass medium, and allow it to harden in oven.

3. Soak slide in warm water, peel off medium, cut into smaller portions as previously marked on the back of the slide, and transfer these portions (same side down) onto adhesive positive control immunohistochemistry slides.
4. Dry slides in oven, dissolve hardened coverglass medium in xylene, re-hydrate slides, and immunostain as usual (including any antigen retrieval procedures). If using pressure cooker antigen retrieval, there is no need to destain the slide, since the pressure cooker antigen retrieval will perform this function.

If material is on **adhesive slides**, an immunostain can be performed on this adhesive slide as usual. (In our experience, tissue transfer immunohistochemistry does not work well on material placed on adhesive slides, since the diagnostic material does not reliably lift off of the original slide). Again, if pressure cooker antigen retrieval is used, it is not necessary to destain the slide before commencing with the immunostain. However, obviously this limits you to performing a single immunostain on the slide.

**TISSUE PROTECTION IMMUNOHISOCHEMISTRY (TPI): A Novel Approach to IHC
on Previously-Stained H&E Slides on Adhesive slides**

We have recently developed a novel procedure that is particularly useful in the evaluation of prostate needle biopsies and similar specimens, where there are frequently more than one H&E level on a slide, but the small focus of interest is not present on deeper sections taken from block. We have term this technique "Tissue Protection Immunohistochemistry (TPI)", and we have employed this technique successfully on numerous occasions. This technique is illustrated graphically in an addendum at the end of the handout.

In order for this technique to be effective, it is critical that the original H&E slides are mounted on adhesive slides. Unlike "standard" tissue transfer immunohistochemistry, the liquid coverglass medium in the TPI method is used to "protect" the H&E sections that are not immunostained. The first step in the method is to select the appropriate H&E level that requires immunostaining, and circle this level on the back of the slide with a diamond tipped pencil. The coverslip is then soaked off, and liquid coverglass medium is placed on the surface of the remaining H&E sections that are NOT to be immunostained. This leaves the section that is to be immunostained uncovered by the liquid coverglass medium. The slide is then subjected to routine antigen retrieval and immunostained as usual. If pressure cooker HIER is used, there is no need to destain the H&E section, since the HIER procedure performs that task effectively. After completion of the immunostain (with appropriate counterstaining in hematoxylin or other stain as desired), the slide is then dehydrated in alcohols and xylene (which dissolves the liquid coverglass medium on the "protected" H&E sections), and coverslipped as usual. The end result is a slide containing one level that is immunostained, and remaining levels that retain their original H&E stain (although there is often slight fading of the H&E stain observed). The chief limitation of this method is that generally only one immunostain can be performed per slide (in contrast to tissue transfer immunohistochemistry, which allows the performance of multiple immunostains from material derived from a single slide. Remember however, that tissue transfer immunohistochemistry requires that the original material be present on **non-adhesive** slides, in contrast to TPI which requires that the original material is on **adhesive** slides).

BLOOD AND BONE MARROW SMEARS

IHC or Flow Cytometry: Which is "Better?"

Most laboratories prefer to use flow cytometry for immunophenotyping of peripheral blood and bone marrow smears in leukemia and lymphoma cases (particularly if they have invested in the equipment and personnel for running a flow cytometer), but this can also be accomplished using immunohistochemical methods (77). In fact, IHC has some advantages over flow cytometry in some cases (particularly for hard-core morphologists), since it allows you to directly visualize the cells in question and determine the presence or absence of specific markers on these cells, unlike flow cytometry. Therefore, in cases where there are only a very small number (e.g., <1%) of "bad" cells in a specimen, these may be more likely to be recognized by IHC rather than flow. However, one requirement for IHC is that a sufficient number of cells must be present on the slides to be assured of a good sample. As such, if the cellularity of a sample is low, it is preferable to study it by flow cytometry rather than IHC. Choice of methodology (IHC vs. flow cytometry) for immunophenotyping is often a matter of personal preference, but I think it is important for people to realize that you do NOT need an expensive, high-tech flow cytometer to provide useful diagnostic immunophenotypic analysis of blood and bone marrow specimens. IHC can provide valuable information in this regard. (Indeed, a significant number of lymphomas are referred to me for immunophenotyping in which flow cytometry was nonspecific or nondiagnostic).

Preparation of Specimens: We use the peroxidase-based method described above (identical to the method used for paraffin section stains) for analysis of blood and bone marrow smears, and it has performed superior to alkaline phosphatase-based methods in our lab. In contrast to cryostat sections, thorough air-drying of blood and bone marrow smears is preferable for optimal morphology (mainly because most hematopathologists are trained on air-dried material). After air-drying, the slides are fixed for 30 seconds in cold (4°C) buffered formalin acetone fixative, pH 6.6, then placed in Phosphate Buffered Saline (PBS), and stored in the refrigerator until they can be stained. As previously mentioned, the method employs a step for quenching of endogenous peroxidase activity (using 0.3% H₂O₂ in PBS with 0.1% sodium azide for 10 minutes) that does not adversely affect TDT or certain fragile B and T cell antigens, unlike some other methods of quenching endogenous peroxidase. Additionally, this step is performed after the secondary (link) antibody incubation, unlike many other methods where the quenching step is performed before the primary antibody incubation. Intensification of the DAB reaction product with copper sulfate is another important element of this method.

In general, I recommend treating this type of material in an identical fashion to air-dried cytologic slides, as described above. Most of the time I suspect that this material will be on non-adhesive slides, and in this situation, I would recommend selecting areas to be stained on one or more slides, and performing tissue transfer immunohistochemistry as described above. This technique allows the performance of multiple immunostains using material from only one slide, and it also enables epitope retrieval procedures to be used, which will enhance immunostain results in air-dried material. If for some reason the smears are on non-adhesive slides, you will generally need one slide for each antibody desired, which may significantly limit the number of different immunostain that can be performed in a particular case.

Fortunately, with the benefit of the currently available antibodies applicable to paraffin-embedded material, many diagnostic problems in hematopathology can be effectively addressed using paraffin sections taken from non-decalcified aspirate clot sections (preferred) or decalcified bone marrow core biopsies.

ARTIFACTS IN IHC

Many different types of artifacts can be observed in IHC. Some of the more common ones that I see are listed below.

1. "Desquamartifact" This is a term that I have coined to describe the most common artifact that I see in IHC, one that I notice every day in our lab. This problem is due to individual squamous cells that flake off (desquamate) from our skin and become lodged on the surface of the glass slides on which the IHC stains are performed. This artifact is usually only noticed on high molecular weight cytokeratin stains (including 34 β E12 and AE1/AE3), and it is easy to understand why. Since squamous cells are loaded with high molecular weight cytokeratin, they stain strongly with that antibody. As a result, any squamous cells that are sitting on the slides will react strongly with cytokeratin, and if they are overlying the tissue section, this will appear as a big "glob" of immunoreactivity lying on top of the section. If you examine these areas closely under high power, it is easy to see the actual squamous cell lying on top of the tissue section. The reactivity of these cells with cytokeratin is often so strong that there is often a "bleeding" of the chromogen into the tissue immediately adjacent to the offending squamous cell. Short of requiring the technologists to wear gloves, hoods, masks, and surgical gowns (obviously somewhat impractical), I am not sure what can be done to address this problem. (Maybe anti-dandruff shampoo is worth a try?)

2. "Bubble" artifacts. On occasion, a small microscopic bubble may find its way onto a tissue section, which in effect "starves" the underlying tissue for reagents. As a result, there will be no immunoreactivity under this "bubble". Since these areas of absent immunoreactivity are circular, they are easy to recognize. Taking care to avoid the production of bubbles in your reagents takes care of this problem. Use of rinsing solutions (PBS or DW) with added Tween 20 (a wetting agent or detergent) may also alleviate this problem, and I have not noticed this problem since we started using Tween added to our rinsing solution.

3. Drying artifacts. Anyone who has worked with IHC for a time has undoubtedly seen this artifact. If the reagents dry out during the incubations, several things can happen, depending on when the slide dried out. The slide may show a total lack of immunoreactivity, or the whole slide may turn a diffuse muddy brown color. If there is only partial drying of the slide, you will often see an irregular band of chromogen deposition that outlines the edge of the dried-out area on the slide. Taking care to assure that your slide trays and incubation surfaces are completely level will help to avoid this problem. In our lab, we rarely see this problem, since our incubation times are short (30 minutes or less), and the use of the "batch" staining method discussed previously eliminates any risk of this artifact, since the sections are totally immersed in reagent.

4. Trapping artifacts. If small microscopic flaps of the tissue section become detached from the slide at some point in the staining procedure, reagents may get trapped under these areas, resulting in a focus of chromogen deposition in this area. This artifact is usually easy to recognize, because it is so focal and the pattern of chromogen deposition generally doesn't correspond to the expected pattern of immunoreactivity in the cells of interest. This is usually a problem with poorly fixed tissue or sclerotic tissue that has been subjected to HIER procedures (pressure cooking or microwaving). For unknown reasons, we have observed that this artifact is much more pronounced when tyramine enhancement techniques are used.

5. Edge artifacts. The edges of tissue sections may display artifactual immunoreactivity, and I think this most likely is a variation of the "trapping artifact" discussed above.

6. Artifacts of inappropriate antibody titers. This has been discussed in great detail in previous portions of this discussion, and if you forgot the details, re-read the section on "true positive" vs. "false positive" staining.

7. Artifacts of poor fixation. Either underfixation or overfixation of a piece of tissue can result in a variable degree of immunoreactivity in different areas of the section of tissue, depending on how well the particular areas of the section have been fixed. In some instances you may see a central area of strong immunoreactivity with a peripheral zone of weak reactivity, secondary to overfixation of the peripheral part, since this part has been penetrated by formalin for a longer time as the formalin works its way to the center of the tissue. Alternatively, underfixation of a piece of tissue often leads to poor immunoreactivity in the central area of the section (since the fixative has not had enough time to penetrate and fix the central area optimally) with stronger immunoreactivity in the peripheral part of the section (since the peripheral areas have been well fixed). Care in cutting the gross tissue to the appropriate size and thickness is important to avoid this artifact, as is attention to fixation time in formalin. The use of vimentin stains can also provide some guidance in assessing the degree of overfixation (antigen damage) that has occurred in different areas of the sections, as discussed previously.

8. Bacterial contamination artifacts. This artifact occurs when bacteria begin to grow in antibody or other solutions, and it appears as finely-granular clumps of chromogen deposition randomly scattered on the surface of the section. This artifact is usually easy to recognize, since the clumps of bacteria are on top of the plane of the section, and they can be recognized as bacteria on high power examination. As you might surmise, this is most commonly observed when using antibody dilutions that have been lying around for a long time. Mixing up fresh aliquots of antibody using clean glassware takes care of this problem. Adding a small pinch of thimerosal (an antibacterial agent) also is effective in preventing this problem.

9. Graphite pencil artifacts. This artifact is not limited to immunostains, as it can be seen any time that lead (graphite) pencils are used to label slides. This artifact results in microscopic flakes of dark black material that get lodged on the slide, and it is due to minuscule particles of graphite that become detached from the label area of the slide and attach to the portion of the slide containing the tissue section (or the blood smear, etc.). Obviously, the solution to this problem is to avoid using graphite pencils to label slides. Solvent-resistant markers work best, and you'll never have to put up with this bothersome artifact.

10. Endogenous biotin artifacts. As previously discussed above in the section on false positive staining, binding of avidin components to endogenous biotin present in tissues or cells may cause artifactual false positive staining. This is most frequently observed in liver and kidney (since these tissues have a high biotin content secondary to numerous mitochondria), where it appears as a granular cytoplasmic chromogen deposition that is noticeable on the stains and also on the negative control specimen. This problem can be eliminated by preincubation of the tissue sections with dilute egg whites (which contain avidin), followed by incubation with reconstituted powdered dry milk or dilute biotin solution, as previously discussed. Many commercial manufacturers of immunostaining reagents market endogenous biotin blocking "kits" that may be employed, for this purpose, but they are much more expensive than using egg whites as a source of avidin. (Obviously, the use of biotin-free detection systems such as PowerVision or EnVision avoids these artifacts).

11. Precipitated DAB artifact. If DAB chromogen is not filtered before use, small bits of precipitated DAB may collect on the surface of the section. The result is similar in appearance to the bacterial contamination artifact discussed above.

12. Mold artifact. Occasionally, we have observed mold growth on some of our control slides, secondary to contaminated slide storage trays, usually with cork bottom inserts. This artifact is easy to recognize once you've seen it, and I will illustrate it during the presentation. Washing slide trays periodically with dilute bleach solution helps to prevent this problem.

CONCLUSION

In conclusion, I think it is important to realize that IHC is a powerful but ancillary technique, and it should be viewed in proper perspective. I also believe it is very important to always keep some "cardinal rules" in mind at all times. Some of these rules are my own, some are borrowed from Dr. Hector Battifora, and some have been taught to me by my mentor in IHC, Dr. Mark Wick. The rules are as follows:

CARDINAL RULES OF IMMUNOHISTOCHEMISTRY

1. Generate a logical differential diagnosis based on the clinical and morphologic findings, and **use panels** of antibodies to narrow the differential diagnosis. Don't succumb to "immunohistochemical guilt" when ordering panels of antibodies for difficult cases.
2. Treat your tissue right (cut it thinly, don't cram the cassettes, and don't let it languish in formalin). Remember, **garbage in, garbage out**.
3. IHC is not perfect and all tumors will not necessarily react as they are "supposed" to. Therefore, if the IHC results don't make any sense, seek an additional opinion. (*Subliminal message: Send the case to me.*)
4. **NEVER use IHC to tell you whether something is benign or malignant.** There are no magic markers of malignancy, and the determination of a tumor's benign or malignant nature is best made by an experienced anatomic pathologist using standard morphologic criteria. There is tremendous potential for disaster when this rule is ignored. (I must confess that occasionally I cheat a little bit on this one).
5. Make your own **Multitumor Block Preparations** and use them. Although multitumor slides are available commercially for about \$13-15.00 per slide, obviously at that price no one can afford to use them in the large numbers necessary for an effective quality control program. For this reason you must make your own (but you can't sell them, they're patented!!).
6. **Do not blindly follow the recommendations of the manufacturer for primary antibody titers**, as they may be way off the mark. Also, remember that **there is no such thing as a "Predilute Ready-to-Use" antibody**.
7. Know the characteristics of a **true positive** and a **false positive** stain. Also, do not interpret poorly fixed tissue, necrotic tissue, or the edges of specimens, as these often exhibit spurious staining.

8. Use **HIER techniques (pressure cooker / vegetable steamer / microwave / hot water bath, etc.) or proteolytic digestion** procedures as needed. Remember that these techniques can also improve IHC staining on cytologic smears as well as paraffin section material. Also, don't assume that every antibody requires antigen retrieval, as a few antibodies perform better without these procedures.

9. If dealing with frozen tissue IHC, **freeze the tissue as rapidly as possible, rapidly fix the sections without any air-drying**, and store the sections in cold PBS until the slides are ready to stain. If dealing with cytologic specimens, do everything in your power to **get a cell block**.

10. **Do not run a stagnant lab.** Continuously and critically evaluate the methods and procedures used on an ongoing basis. Experiment with new methods, fixatives, reagents, techniques, and modifications. That is the only way that your laboratory will grow and improve with age.

References

1. Arber JM, Arber DA, Jenkins KA, Battifora H: Effect of decalcification and fixation in paraffin-section immunohistochemistry. *Applied Immunohistochemistry* 4(4): 241-248, 1996.
2. Matthews JB, Mason GI: Influence of decalcifying agents on the immunoreactivity of formalin-fixed paraffin-embedded tissue. *Histochem J* 1984;16:771-787.
3. Mukai K, Yoshimura S, Anzai M: Effects of decalcification on immunoperoxidase staining. *Am J Surg Pathol* 1986;10:413-419.
4. Athanasou NA, Quinn J, Heryet A et al: Effect of decalcification agents on immunoreactivity of cellular antigens. *J Clin Pathol* 1987;40:874-878.
5. Arber JM, Arber DA, Jenkins KA et al: Effect of decalcification and fixation in paraffin-section immunohistochemistry. *Appl Immunohistochem* 1996;4(4):241-248.
6. Battifora H, Kopinski M: The influence of protease digestion and duration of fixation on the immunostaining of keratins: A comparison of formalin and ethanol fixation. *The Journal of Histochemistry and Cytochemistry* 34:1095-1100, 1986.
7. Pinkus GS, O'Connor EM, Etheridge CL et al: Optimal immunoreactivity of keratin proteins in formalin-fixed, paraffin-embedded tissue requires preliminary trypsinization. An immunoperoxidase study of various tumors using polyclonal and monoclonal antibodies. *J Histochem Cytochem* 1986;34:1095-1100.
8. Andrade RE, Hagen KA, Swanson PE, et al: The use of proteolysis with ficin, for immunostaining of paraffin sections. A study of lymphoid, mesenchymal, and epithelial determinants in human tissues. *Am J Clin Pathol* 1988;90(1):33-39.
9. Ordonez NG, Manning JT, Brooks TE: Effect of trypsinization on the immunostaining of formalin-fixed, paraffin-embedded tissues. *Amer J Surg Pathol* 1988;12(2):121-129.
10. Shi S-R, Key ME, Kalra KL: Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave heating of tissue sections. *J Histochem Cytochem* 1991;39:741-748.
11. Catoretto G, Pileri S, Parravicini C et al: Antigen unmasking on formalin-fixed paraffin-embedded sections. *J Pathol* 1993;171:83-98.
12. Taylor CR, Shi SR, Chaiwun B et al: Strategies for improving the immunohistochemical staining of various intranuclear prognostic markers in formalin-paraffin sections: Androgen receptor, estrogen receptor, progesterone receptor, p53 protein, proliferating cell nuclear antigen, and Ki-67 antigen revealed by the antigen retrieval technique. *Human Pathology* 25:263-270, 1994.
13. Leong ASY, Milios J: An assessment of the efficacy of the microwave antigen-retrieval procedure on a range of tissue antigens. *Applied Immunohistochemistry* 1(4): 267-274, 1993.
14. Gown AM, de Wever N, Battifora H: Microwave-based antigenic unmasking. A revolutionary technique for routine immunohistochemistry. *Applied Immunohistochemistry* 1(4):256-266, 1993.
15. Beckstead, JH: Improved antigen retrieval in formalin-fixed, paraffin-embedded tissue. *Applied Immunohistochemistry* 2(4): 274-281, 1994.
16. Cattoretto G, Suurmeijer AJH: Antigen unmasking on formalin-fixed paraffin-embedded tissues using microwaves: A Review. *Advances in Anatomic Pathology* 2(1): 2-9, 1995.
17. Leong ASY, Milios J, Leong FJ: Epitope retrieval with microwaves. A comparison of citrate buffer and EDTA with three commercial retrieval solutions. *Applied Immunohistochemistry* 4(3): 201-207, 1996.
18. Taylor CR, Shi SR, Cote RJ: Antigen retrieval for immunohistochemistry. Status and need for greater standardization. *Applied Immunohistochemistry* 4(3): 144-166, 1996.
19. Suurmeijer AJH, Boon ME: Notes on the application of microwaves for antigen retrieval in paraffin and plastic sections. *Eur J Morphol* 1993;31:144-150.
20. Shi S-R, Gu J, Kalra KL et al: Antigen retrieval technique: A novel approach to immunohistochemistry on routinely processed tissue sections. *Cell Vision* 1995;2(1): 7-22.
21. Shi S-R, Cote RJ, Young LL et al: Antigen retrieval immunohistochemistry: Practice and development. *J Histotechnol* 1997;20(2):145-154.
22. Shi S-R, Cote RJ, Chaiwun B et al: Standardization of immunohistochemistry based on antigen retrieval technique for routine formalin-fixed tissue sections. *Appl Immunohistochem* 1998;6(2):89-96.

23. Elias J, Margiotta M: Low temperature antigen restoration of steroid hormone receptor proteins in routine paraffin sections. *J Histotechnol* 1997;20(2):155-158.
24. Linden MD: Immunohistochemistry using hot plate heat-induced epitope retrieval: A simple and cost-effective alternative to microwave techniques (abstract). *Am J Clin Pathol* 1998;110:544-545.
25. Pasha T, Montone KT, Tomaszewski JE: Nuclear antigen retrieval utilizing steam heat (abstract). *Lab Invest* 1995;72:4167-4168.
26. Bankfalvi A, Navabi H, Bier B et al: Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. *J Pathol* 1994;174:223-228.
27. Pons C, Costa I, von Schilling B et al: Antigen retrieval by wet autoclaving for p53 immunostaining. *Appl Immunohistochem* 1995;3(4):265-267.
28. Miller K, Auld J, Jessup E et al: Antigen unmasking in formalin-fixed routinely processed paraffin wax-embedded sections by pressure cooking: A comparison with microwave oven heating and traditional methods. *Adv Anat Pathol* 1995;2(1):60-64.
29. Miller RT, Estran C: Heat-induced epitope retrieval with a pressure cooker. Suggestions for optimal use. *Appl Immunohistochem* 1995;3(3):190-193.
30. Morgan JM, Navabi H, Schmid KW, Jasani B: Possible role of tissue-bound calcium ions in citrate-mediated high temperature antigen retrieval. *Journal of Pathology* 174(4): 301-307, 1994.
31. Yaziji H, Gown AM: Divalent metal ion chelation is a universal underlying mechanism in heat induced epitope retrieval (abstract). *Modern Pathology* 10(1): 186A, 1997.
32. Shi SR, Imam A, Young RJ et al: Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *The Journal of Histochemistry and Cytochemistry* 43:193-201, 1995.
- 32a. Biddolph SC, Jones M: low-temperature, heat-mediated antigen retrieval (LTHMAR) on archival lymphoid sections. *Applied Immunohistochemistry and Molecular Morphology* 7 (4): 289-293, 1999.
33. Portiansky EL, Gimeno EJ: A new epitope retrieval method for the detection of structural cytokeratins in the bovine prostatic tissue. *Applied Immunohistochemistry* 4(3): 208-214, 1996.
- 33a Hammoud M, Van Noorden S: Failure of Ultrasonic Vibration As a Means of Antigen Retrieval in Routine Diagnostic Immunocytochemistry. *Applied Immunohistochemistry & Molecular Morphology* 8 (3): 249-255, 2000.
34. Brynes RK, McCourty A, Tamayo R et al: Demonstration of cyclin D1 (Bcl-1) in mantle cell lymphoma. Enhanced staining using heat and ultrasound epitope retrieval. *Applied Immunohistochemistry* 5(1): 45-48, 1997.
35. Miller RT, Kubier P: Blocking of endogenous avidin-binding activity in immunohistochemistry: The use of egg whites. *Applied Immunohistochemistry* 5(1): 63-66, 1997.
36. Fan Z, Clark V, Nagle RB: An evaluation of enzymatic and heat epitope retrieval methods for the immunohistochemical staining of the intermediate filaments. *Applied Immunohistochemistry* 5(1): 49-58, 1997.
37. Rodriguez-Soto J, Warnke RA, Rouse RV: Endogenous avidin-binding activity in paraffin-embedded tissue revealed after microwave treatment. *Applied Immunohistochemistry* 5(1): 59-62, 1997.
38. McCluggage WG, Maxwell P, Patterson A et al: Immunohistochemical staining of hepatocellular carcinoma with monoclonal antibody against inhibin. *Histopathology* 1997;30:5168-522.
39. Iezzoni JC, Mills SE, Pelkey TJ et al: Inhibin is not an immunohistochemical marker for hepatocellular carcinoma. An example of the potential pitfall in diagnostic immunohistochemistry caused by endogenous biotin. *Am J Clin Pathol* 111:229-234, 1999.
40. Prento P, Lyon H: Commercial formalin substitutes for histopathology. *Biotech Histochem* 1997;72(5):273-282.
41. Meyer R, Niedobitek F, Wenzelides K: Experiences with the NoToX formalin substitute solution. (article in German) *Pathologie* 1996;17(2):130-132.
42. Vince DG, Tbakhi T, Gaddipati A et al: Quantitative comparison of immunohistochemical staining intensity in tissues fixed in formalin and Histochoice. *Anal Cell Pathol* 1997;15(2):119-129.
43. Bostwick DG, al Annouf N, Choi C: Establishment of the formalin-free surgical pathology laboratory. Utility of an alcohol-based fixative. *Arch Pathol Lab Med* 1994;118(3):298-302.
44. Dorfman DM, Shahsafaei A: Usefulness of a new CD5 antibody for the diagnosis of T-cell and B-cell lymphoproliferative disorders in paraffin sections. *Mod Pathol* 1997;10(9):859-863
- 44a. Facchetti F et al: Omit Iodine and CD30 Will Shine: a Simple Technical Procedure to Demonstrate the CD30 Antigen on B5-fixed Material. (Letter to the Editor). *American Journal of Surgical Pathology* 24 (2): 320-322, 2000

45. Abbodonzio SL, Allred C, Lampkin S et al: Enhancement of immunoreactivity among lymphoid malignant neoplasms in paraffin-embedded tissues by refixation in zinc sulfate-formalin. *Arch Pathol Lab Med* 1991;115:31-33.
46. Arnol MM, Srivastava S, Fredenburgh J et al: Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens. *Biotech Histochem* 1996;71(5):224-230.
47. Dapson RW: Fixation for the 1990's: A review of needs and accomplishments. *Biotech Histochem* 1993;68(2):75-82.
48. Henderson C: Aminoalkylsilane: An inexpensive, simple preparation for slide adhesion. *J Histochem Cytochem* 12(2):123-124, 1989.
49. Iezzoni JC, Manahan LJ, Park CS, Brigati DJ: Recent advances in automated immunocytochemistry. *The Journal of Histotechnology* 16(1):39-49, 1993.
50. Yorukoglu K, Cingoz S: Epitope retrieval technique: A simple modification that reduces staining time (Letter to the Editor). *Applied Immunohistochemistry* 5(1): 71, 1997.
51. Li C-Y, Zeismer SC, Lazcano-Villareal O: Use of azide and hydrogen peroxide as an inhibitor for endogenous peroxidase in the immunoperoxidase method. *The Journal of Histochemistry and Cytochemistry* 35(12):1457-1460, 1987.
52. Aratake Y, Tamura K, Kotani T, Ohtaki S: Application of the avidin-biotin complex method for the light microscopic analysis of lymphocyte subsets with monoclonal antibodies on air-dried smears. *Acta Cytologica* 32:117-122, 1988.
53. Tacha D, McKinney L: Casein reduces nonspecific background staining in immunolabeling techniques. *The Journal of Histotechnology* 15:127-132, 1992.
54. Wood GS, Warnke R: Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems. *The Journal of Histochemistry and Cytochemistry* 29(10): 1196-1204, 1981.
55. Miller RT, Kubier P, Reynolds B et al: Blocking of endogenous avidin-binding activity in immunohistochemistry: The use of skim milk as an economical and effective substitute for commercial biotin solutions. *Applied Immunohistochemistry & Molecular Morphology* 7(1):63-65, 1999.
56. Bindl JM, Warnke RA: Advantages of detecting monoclonal antibody binding to tissue sections with biotin and avidin reagents in Coplin jars. *American Journal of Clinical Pathology* 85(4): 490-493, 1986.
57. Ciocca DR, Adams DJ, Bjercke RJ et al: Monoclonal antibody storage conditions and concentration effects on immunohistochemical staining. *The Journal of Histochemistry and Cytochemistry* 31:691-696, 1983.
58. Boenisch T: Diluent Buffer Ions and pH: Their Influence on the Performance of Monoclonal Antibodies in Immunohistochemistry. *Applied Immunohistochemistry & Molecular Morphology* 7(4): 300-306, 1999.
59. Miller, RT: Multitumor "Sandwich" Blocks in Immunohistochemistry. Simplified Method of Preparation and Practical Uses. *Applied Immunohistochemistry* 1(2):156-159, 1993.
60. Sickel JZ, Sant'Agnes PA: Anomalous immunostaining of 'optically clear' nuclei in gestational endometrium. A potential pitfall in the diagnosis of pregnancy-related herpesvirus infection. *Archives of Pathology and Laboratory Medicine* 118:831-833, 1994.
61. Wiczorek R, Stover R, Sebenik M: Nonspecific nuclear immunoreactivity after antigen retrieval using acidic or basic solutions. *J Histotechnol* 20(2):139-143, 1997.
62. Chang KL, Arber DA, Weiss LM: CD20: A review. *Applied Immunohistochemistry* 4(1): 1-15, 1996.
63. Butz WR, Clark KC, Miller RT: Improved manual immunohistochemistry employing orbital mixing of reagents during incubations. *Applied Immunohistochemistry* 2(1): 65-67, 1994.
64. Sofroniew MV, Schrell U: Long term storage and regular repeated use of diluted antisera in glass staining jars for increased sensitivity, reproducibility, and convenience of single- and two-color light microscopic immunocytochemistry. *The Journal of Histochemistry and Cytochemistry* 30:504-511, 1982.
65. Bindl JM, Warnke RA: Advantages of detecting monoclonal antibody binding to tissue sections with biotin and avidin reagents in Coplin jars. *American Journal of Clinical Pathology* 85(4): 490-493, 1986.
66. Tacha D, Brown M, Galey WT: A two step immunoperoxidase detection system with repeated use of diluted antibodies for bulk staining of slides. *The Journal of Histotechnology* 13(1):15-20, 1990.
67. Pfeiffer P, Grabau DA, Nielsen HT, Clausen PP: Immunohistochemical bulk staining of slides using a rack peroxidase-labeled streptavidin-biotin technique. *Applied Immunohistochemistry* 4(2): 135-138, 1996.

68. Kamino H, Tam ST: Immunoperoxidase technique modified by counterstain with azure B as a diagnostic aid in evaluating heavily pigmented melanocytic neoplasms. *Journal of Cutaneous Pathology* 18:436-439, 1991.
69. Sayadi H, Fitzgibbon JF, Ritter JH et al: Azure B as a counterstain in the immunohistological evaluation of heavily pigmented nevocytic lesions. *Applied Immunohistochemistry* 3(4): 268-271, 1995.
- 69a. Kligora CJ, Fair KP, Clem MS et al: A comparison of melanin bleaching and azure Blue counterstaining in the immunohistochemical diagnosis of malignant melanoma. *Mod Pathol* 12(12):1143-1147, 1999.
70. Bobrow MN, Harris TD, Shaughnessy KJ et al: Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays. *J Immunological Methods* 125:279-285, 1989.
71. Adams JC: Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *J Histochem Cytochem* 40:1457-1463, 1992.
72. Merz H, Malisius R, Mannweiler S et al: ImmunoMax. A maximized immunohistochemical method for the retrieval and enhancement of hidden antigens. *Lab Invest* 73(1):149-156, 1995.
73. Jacobs W, Dhaene K, Van Marck E: Tyramine-amplified immunohistochemical testing using "homemade" biotinylated tyramine is highly sensitive and cost effective. *Arch Pathol Lab Med* 122:642-643, 1998.
74. Miller, RT: Multitumor "Sandwich" Blocks in Immunohistochemistry. Simplified Method of Preparation and Practical Uses. *Applied Immunohistochemistry* 1(2):156-159, 1993.
75. Battifora H: Assessment of antigen damage in immunohistochemistry. The vimentin internal control. *American Journal of Clinical Pathology* 96:669-671, 1991.
76. Miller RT: Immunohistochemistry in the community practice of pathology. Part 1: General considerations, technical factors, and quality assurance. *Laboratory Medicine* 22:457-464, 1991.
77. Miller RT, Groothuis CG: Improved avidin-biotin immunoperoxidase method for terminal deoxyribonucleotidyl transferase and immunophenotypic characterization of blood cells. *American Journal of Clinical Pathology* 93:670-674, 1990.
78. Sherman ME, Jimenez-Joseph D, Gangi MD et al: Immunostaining of small cytologic specimens. Facilitation with cell transfer. *Acta Cytologica* 38(1):18-22, 1994.
79. Mehta P, Battifora H: How to do multiple immunostains when only one tissue slide is available. The "peel and stick" method. *Applied Immunohistochemistry* 1(4): 297-8, 1993.
80. Leung SW, Bedard YC: Immunocytochemical Staining on ThinPrep Processed Smears. *Modern Pathology* 9 (3): 3004-3006, 1996.
81. Kaplan MA, Segura AM, Wang HH et al: Evaluation of Cytolyt and PreservCyt Aservatives for Immunocytochemistry for Cytokeratin in Fine Needle Aspiration. *Applied Immunohistochemistry* 6 (1): 23-29, 1998.
82. Leong AS-Y, Suthipinatwong C, Vinyuvat S: Immunostaining of Cytologic Preparations: a Review of Technical Problems. *Applied Immunohistochemistry & Molecular Morphology* 7 (3): 214-220, 1999.
83. Vyberg M, Nielsen S: Dextran polymer conjugate two-step visualization system for immunohistochemistry. A comparison of EnVision+ with two three-step avidin-biotin techniques. *Appl Immunohistochem* 1998;6(1):3-10.
84. Shi S-R, Guo J, Cote RJ et al: Sensitivity and Detection Efficiency of a Novel Two-Step Detection System (PowerVision) for Immunohistochemistry. *Applied Immunohistochemistry & Molecular Morphology* 7 (3): 201-208, 1999.

Titration Studies in Immunohistochemistry

Rodney T. Miller, M.D.

Director of Immunohistochemistry, ProPath Laboratory, Inc., Dallas, Texas

Introduction

Immunohistochemistry is an extremely powerful tool in diagnostic pathology, but in order for it to be useful, the conditions of the staining method must be optimized to ensure that the resulting stains are reliable and accurate. A critical factor in optimizing the staining conditions is to make certain that the appropriate concentration of primary antibody (i.e., the appropriate titer) is used. This is necessary because the characteristics of the antigen-antibody interaction require suitable relative concentrations of the antigen and antibody in order to optimize the binding between the two. If there is too much antibody in solution (relative to the amount of antigen), the antigen and antibody will be unable to bind effectively, resulting in a poor interaction, and a poor immunostain. In this situation, the resulting stain usually shows high levels of nonspecific "background" staining or displays a false positive uniform cytoplasmic "blush" in tumor cells. If there is too little antibody present, the stain will be too weak. **Remember, there is no such thing as a "Predilute, Ready-To-Use" antibody!!!** I believe that the most common reason for generation of unreliable, uninterpretable, or misleading results in substandard immunohistochemistry labs is the failure of these labs to perform the needed titration studies, or performing them incorrectly. Of course, this never happens at the ProPath Immunohistochemistry Laboratory.

Definitions

The word "titer" refers to the extent to which a particular solution is diluted by adding another solution. In our situation, we will be diluting our primary antibodies by adding our standard antibody diluent solution (which is PBS with 2% bovine serum albumin).

Titers are usually expressed as a ratio. For example, a titer of "1:10" (1 to 10) means that a given volume of the initial solution (the primary antibody) is further diluted (by adding the diluent) so that the final volume is now 10 times more than the volume of the initial solution that we started with. If we are starting with 1 ml of antibody and want to dilute all of it by 1:10, we would place the 1 ml of antibody into a test tube (that holds at least 10 ml), and add 9 ml of diluent, so that our new final volume is 10 ml. If we wanted to make a titer of 1:100, we would take the 1 ml of antibody and add 99 ml of diluent, so that our new final volume is now 100 ml.

The above example is for illustration purposes only, since **the antibodies are very expensive**, and we would almost never dilute an entire 1 ml of primary antibody, since that would often consume the entire vial. So, in actual practice, we will try to take only as much primary antibody as is needed to make a small amount (usually 1 ml or so) of final solution. For example, to make 1 ml (final volume) of a 1:100 titer of a primary antibody, we would take 0.01 ml of primary antibody, and add diluent to a total final volume of 1 ml (so we would be adding 0.99 ml of diluent). Because of the small volumes of primary antibodies used to prepare our titers, special calibrated pipettes are needed to prepare the various titers.

Units of Measurement

A thorough understanding of the units of measurement employed is necessary in order to perform titration studies accurately, and this is often a confusing and difficult aspect to master

when first learning to perform titration studies. One commonly used measure is described as " λ " (lambda), which is the same as " μ l" (microliter). Both " λ " and " μ l" are equal to 0.001 ml (1/1000 of a ml). Some conversion factors are listed below:

$$\begin{array}{rcl} 1 \lambda & = & 0.001 \text{ ml} \\ 10 \lambda & = & 0.01 \text{ ml} \end{array} \qquad \begin{array}{rcl} 100 \lambda & = & 0.1 \text{ ml} \\ 1000 \lambda & = & 1 \text{ ml} \end{array}$$

Practical Titering in the Immunohistochemistry Lab

The range of useful titers in immunohistochemistry is very broad, ranging from undiluted reagents (referred to as "neat") to reagents that are titered out to 1:20,000,000.

As previously mentioned, due to the high cost of the primary antibodies used, a high priority must be placed on avoiding waste of these reagents. Because of this, the initial volumes that we will be dealing with will be quite small.

When first starting to titer a primary antibody, we will usually try to have an initial final volume of 0.5 ml or 1 ml. For example, if we want to prepare a titer of an antibody at 1:100, we can do either of the following:

For a final volume of 0.5 ml:

Take 5 λ of primary antibody, add 0.495 ml of diluent

For a final volume of 1.0 ml:

Take 10 λ of primary antibody, add 0.99 ml of diluent

"No-Brainer" Titering Cheat Sheet

Even people who do titering on a daily basis may experience a "brain fart", and may screw up the preparation of a titer or series of titers. For this reason, I have prepared the following "No-Brainer" Titering Cheat Sheet for those days when brain gas is a problem.

<u>Final Volume</u>	<u>Titer</u>	<u>Amount of Antibody</u>	<u>Amount of Diluent</u>
1.0 ml	1:10	100 λ or 0.1 ml	0.9 ml
1.0 ml	1:20	50 λ or 0.05 ml	0.95 ml
1.0 ml	1:25	40 λ or 0.04 ml	0.96 ml
1.0 ml	1:40	25 λ or 0.025 ml	0.975 ml
1.0 ml	1:50	20 λ or 0.02 ml	0.98 ml
1.0 ml	1:80	12.5 λ or 0.0125 ml	0.9875 ml
1.0 ml	1:100	10 λ or 0.01 ml	0.99 ml
1.0 ml	1:200	5 λ or 0.005 ml	0.995 ml
1.0 ml	1:250	4 λ or 0.004 ml	0.996 ml
2.0 ml	1:10	200 λ or 0.2 ml	1.8 ml
2.0 ml	1:20	100 λ or 0.1 ml	1.9 ml
2.0 ml	1:25	80 λ or 0.08 ml	1.92 ml
2.0 ml	1:40	50 λ or 0.05 ml	1.95 ml
2.0 ml	1:50	40 λ or 0.04 ml	1.96 ml
2.0 ml	1:80	25 λ or 0.025 ml	1.975 ml
2.0 ml	1:100	20 λ or 0.02 ml	1.98 ml
2.0 ml	1:200	10 λ or 0.01 ml	1.99 ml
2.0 ml	1:250	8 λ or 0.008 ml	1.992 ml
2.0 ml	1:400	5 λ or 0.005 ml	1.995 ml
2.0 ml	1:500	4 λ or 0.004 ml	1.996 ml

Preparation of Highly Dilute Titrers

On occasion, it will be necessary to prepare highly dilute titers of some antibodies (e.g., final titers of 1:50,000, 1:500,000 or even 1:1 million, etc.). In this situation, it is best to first prepare a "stock" dilution of 1:50, 1:100, 1:200, or thereabouts; and to then prepare the final dilution by further dilutions of this "stock" dilution. This will result in less waste (since smaller final volumes will be able to be used), and the accuracy of the final highly dilute solution will be much better than if you tried to make the highly dilute solution in only one step. For example, to make a 1:1 million dilution in one step, you would need 1 λ of antibody in 1 liter of diluent. One λ is such a tiny amount that there are few pipettes that can accurately deliver this small amount. Furthermore, it would be wasteful to make 1 liter of diluted antibody, since it would take a long time to use.

Serial Dilution Studies

After purchasing a new antibody, it is necessary to determine its optimal titer before putting it into diagnostic use. In this situation, a series of stains is performed, with the primary antibody used at a number of different titers (different concentrations or different dilutions).

The easiest way to perform this task is to perform serial 2-fold dilutions of the most concentrated titer you wish to test (which will be the first one you prepare). For example, a set of serial dilution studies might include the primary antibody used at titers of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, and 1:6400 (for a total of 7 stains performed). The resulting stains are viewed, and the "best" stain is then chosen as the optimal titer for that particular antibody, which will then be used for all further diagnostic cases unless otherwise specified.

Preparation of Serial Dilutions

Preparation of serial dilutions is not difficult. Generally, it is easiest to start with an initial volume of 1 ml of solution. For example, if we are planning to do a set of serial dilution studies beginning at 1:100 (followed by 1:200, 1:400, 1:800, etc.), the first thing to do is to prepare 1 ml of a 1:100 dilution, which for the sake of discussion we will name test tube "A". Next, take 0.5 ml of this solution, and place it in another test tube (named "B"), and then add 0.5 ml of diluent to test tube "B" and mix. Test tube "B" now has a 1:200 dilution of the primary antibody. Next, take 0.5 ml from test tube "B" and add it to another test tube ("C"). Add 0.5 ml of diluent to test tube "C", and we now have 1 ml of 1:400 primary antibody solution in test tube "C". To prepare the next dilution (1:800), take 0.5 ml out of test tube "C", and place it in another test tube ("D"). Now add 0.5 ml of diluent to test tube "D", and we now have 1 ml of 1:800 dilution of primary antibody in test tube "D". Further serial dilutions are made by repeating the above procedure.

Again, the high cost of these reagents needs to be emphasized, so we do not want to waste any of this material. Therefore, we rarely throw the stuff out. This results in millions of little test tubes in the refrigerator, but there is no good way around this problem that I know of. The importance of proper and complete labeling of the test tubes is obvious.

I hope this information is useful to those who are in the process of learning to perform titration studies in immunohistochemistry. These studies are critical, and will need to be performed on a frequent basis in any immunohistochemistry lab that desires to produce consistently accurate, reliable, and sensitive stains.

NOTE: As an additional aid to titration studies, I have prepared a computer spreadsheet document that allows the determination of quantities of antibody and diluent needed to make any titration, simply by "filling in the blanks". This spreadsheet can also be used to determine how much of an antibody of known titer to add to another solution of known titer to reach a desired titer (for example, if you have a solution at 1:500 and you want to make it 1:300). I will be happy to send anyone a copy of this spreadsheet by E-mail or on a floppy disc. If you would like a copy, E-mail me and I'll send you the file by return E-mail. Alternatively, send me a brief note along with a blank IBM-formatted floppy disc and a postage-paid self addressed mailing container, and I will return the file to you on the disc. I have it available in either Microsoft Excel 97 or Microsoft Works for Windows 4.0. Please specify which software you have. (Sorry, I do not have it available for Macintosh computers). -RTM January 2000

USEFUL INTERNET SITES FOR IMMUNOHISTOCHEMISTRY

www.appliedimmuno.org Website of the Society for Applied Immunohistochemistry

<http://ipox.stanford.edu/cgi-bin/ipox>

Fabulous IHC literature database developed and maintained by Dr. R. Rouse and colleagues from Stanford. For username, type "guest": for password, leave it blank (no password required)

<http://www.ncbi.nlm.nih.gov/PubMed/>

PubMed: The National Library of Medicine's free search service to access the 9 million citations in MEDLINE and Pre-MEDLINE (with links to participating on-line journals), and other related databases.

<http://www.medmatrix.org/index.stm/>

Medical Matrix: A very useful guide to medical resources on the internet, with links to a number of Medline and other databases, including several of those listed below.

<http://igm.nlm.gov/>

Internet Grateful Med: Another National Library of Medicine free search service to access MEDLINE and a number of other medical information databases.

<http://www.avicenna.com/>

AVICENNA: an excellent free MEDLINE/MEDLARS Database search engine for medical information.

<http://immuno.hypermart.net/>

IMMUNOHISTOCHEMISTRY HOME PAGE: A site that has a number of useful and interesting IHC links.

See also: Turbett GR and Sellner LN: A comprehensive guide to pathology resources available on the internet. *Advances in Anatomic Pathology* 3(5):313-335, 1996.

Please E-mail me with other sites that you think are useful and I'll add them to the list.

rmiller@propathlab.com

ProPath Laboratory, Inc.

Immunohistochemistry Division

8267 Elmbrook, Suite 100, Dallas, Texas 75247 (214) 638-2000, ext. 2037

www.propathlab.com E-Mail: rmiller@propathlab.com

Giemsa / Hematoxylin Counterstain Procedure

(An aid to distinguish melanin from Diaminobenzidine chromogen when working with tissues or smears containing abundant melanin pigment. This counterstain will turn the melanin granules a dark blue-green-black color that is easily distinguished from the brown color of the DAB chromogen.)

- 1. Hydrate slides to distilled water.**
- 2. Place slides in Giemsa stain for 2 minutes at room temp (we use Reagent B Blue Stain from Wescor, order # SS-035B)**
- 3. Rinse in Tap Water**
- 4. Stain in Hematoxylin for 15 seconds (we use Hematoxylin 1 from Richard-Allen Scientific, order # 7221)**
- 5. Rinse in tap water**
- 6. Dehydrate, clear and coverslip.**

If you don't have the same Giemsa or Hematoxylin listed above, just experiment a little bit with your current reagents, and eventually you should get something that works for you.

Revised 11-3-2000

Rodney T. Miller, MD
Director of Immunohistochemistry

Pertinent References:

1. Kamino H, Tam ST: Immunoperoxidase technique modified by counterstain with azure B as a diagnostic aid in evaluating heavily pigmented melanocytic neoplasms. *Journal of Cutaneous Pathology* 18:436-439, 1991.
2. Sayadi H, Fitzgibbon JF, Ritter JH et al: Azure B as a counterstain in the immunohistological evaluation of heavily pigmented nevocytic lesions. *Applied Immunohistochemistry* 3(4): 268-271, 1995.
3. Kligora CJ, Fair KP, Clem MS et al: A comparison of melanin bleaching and azure Blue counterstaining in the immunohistochemical diagnosis of malignant melanoma. *Mod Pathol* 12(12):1143-1147, 1999.

ProPath Laboratory, Inc.

Immunohistochemistry Division

8267 Elmbrook, Suite 100, Dallas, Texas 75247 (214) 638-2000, ext. 2037

www.propathlab.com E-Mail: rmiller@propathlab.com

Henderson's method for preparing silanized slides

REFERENCE: Henderson C: Aminoalkylsilane: An inexpensive, simple preparation for slide adhesion. *J Histochem Cytochem* 12(2):123-124, 1989.

Solution Used: 2% aminoalkylsilane (AAS) in dry acetone

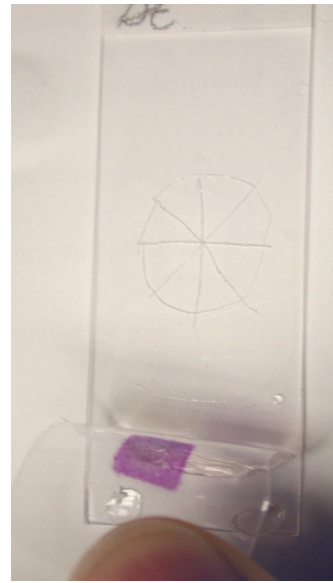
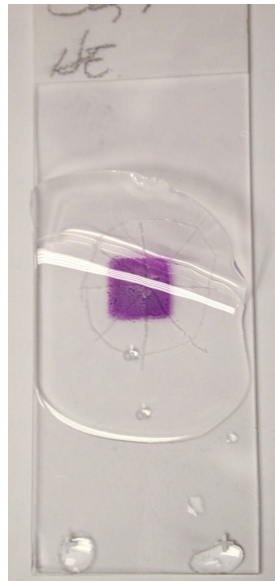
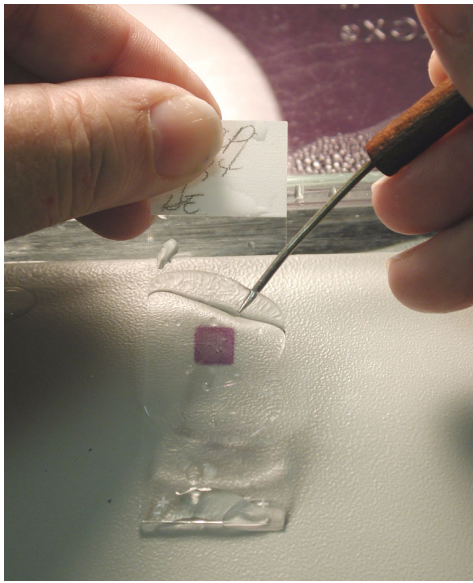
3-aminopropyltriethoxysilane (AAS) (Sigma A-3648)	5 ml
Dry acetone (99.5% pure)	250 ml

Since aminoalkylsilane is moisture sensitive, store at 4 degrees C in a tightly closed container of anhydrous calcium sulfate (a 100 ml bottle of Sigma AAS fits nicely into a 1/3 full, 1 lb. container of 10-20 mesh "Drierite").

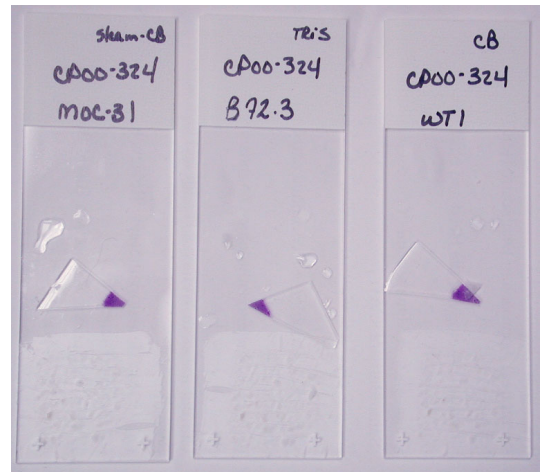
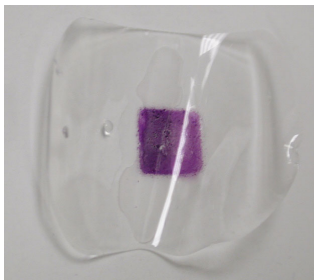
Always make up the 2% working solution just prior to use, use only once, and discard.

PROCEDURE AS RECOMMENDED BY HENDERSON:

1. Dip slides in 2% AAS (250 ml) 10 seconds
2. Rinse in deionized water (1000 ml) 5 dips
3. Rinse in deionized water (1000 ml) 5 dips
4. Rinse in deionized water (1000 ml) 5 dips
5. Air dry overnight
6. Store in slide boxes at room temperature



The use of a sharp probe may also assist in peeling the material off of the slide, as shown above.



4. Once the material is peeled off it is cut into smaller pieces as previously marked (a single-edged razor blade works well), and then placed (same side down) onto adhesive positive control slides, as shown on the right.

These are then dried in the oven, deparaffinized in xylene (which dissolves the liquid coverglass medium), and immunostained as usual.

5. Three of the eight completed immunostains are shown at the right, along with a spare H&E-stained cytospin slide.



Rodney T. Miller, M.D.
Director of Immunohistochemistry
May, 2001

ProPath Laboratory, Inc.
Immunohistochemistry Division
 8267 Elmbrook, Suite 100, Dallas, Texas 75247-4009
 Lab (214) 638-2000, ext 2037, Fax (214) 237-1770
<http://www.propathlab.com/> E-mail: rmiller@propathlab.com

TISSUE PROTECTION IMMUNOHISTOCHEMISTRY

(Excerpted from Kubier P, Miller RT; "Tissue Protection Immunohistochemistry (TPI): a Useful Adjunct in the Interpretation of Prostate Biopsies and Other Select Cases Where Immunostains are Needed on Minute Lesions.", submitted for publication July 2001)

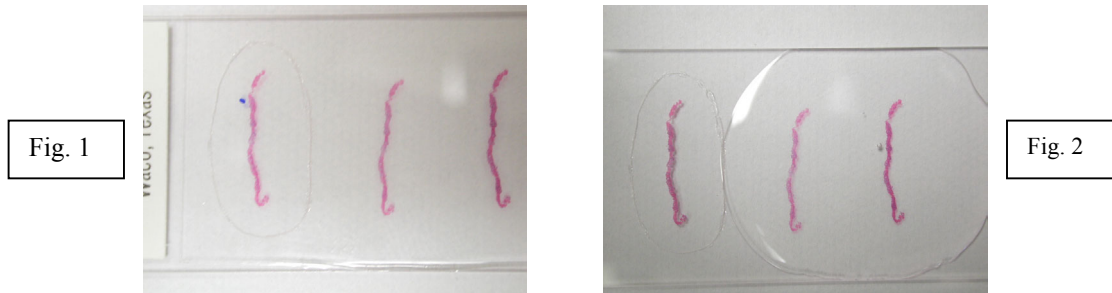


Figure 1 shows an H&E slide of a needle biopsy of prostate, which has been mounted on an adhesive slide (a requirement for TPI immunohistochemistry). The biopsy contained a minute suspicious area (indicated by a small dot), that was only present on this particular slide, and disappeared in deeper cuts. A high molecular weight cytokeratin immunostain will be performed on the left-most section of this slide. The first step in TPI is to mark the section to be immunostained on the back surface of the slide with a diamond tipped pencil (Figure 1). After removing the coverslip, liquid coverglass media is applied to cover the 2 H&E levels which will NOT be immunostained (to "protect" these H&E levels, Figure 2). At this point, the slide is subjected to pressure cooker epitope retrieval and immunostained for high molecular weight cytokeratin as usual.

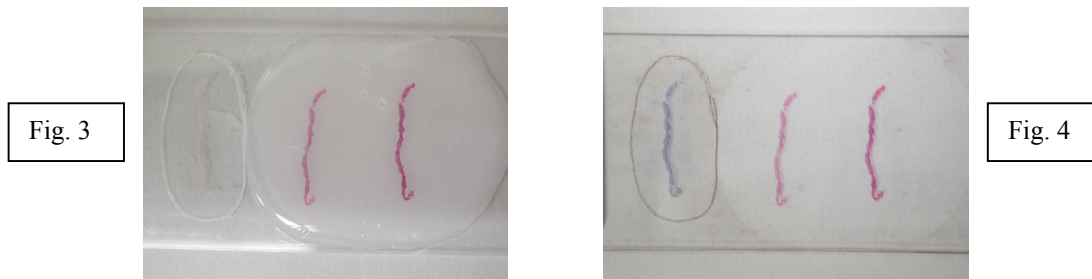


Figure 3 shows the appearance of the slide immediately after it has been removed from the pressure cooker. The pressure cooker epitope retrieval step has de-stained the left-most section, although the 2 remaining H&E sections have been "protected" by the liquid coverglass media, and still retain their H&E stain. Figure 4 shows a completed TPI immunostain. Routine counterstaining is employed, and the coverglass media will dissolve during the final deparaffinization step prior to coverslipping. The two "protected" H&E sections on the right side of the slide are unaltered, and on the left there is a section that has been immunostained with high molecular weight cytokeratin. This arrangement makes it very convenient to compare and correlate the appearance of the immunostain and the corresponding H&E sections.