Making Immunohistochemistry Work for You

IHC is nearly ubiquitous in histology today. If the procedures are not performed on site, specimens are sent to labs specializing in this work. Our field truly has been revolutionized by this technology, yet the complexities of procedures and variations among specimens occasionally frustrate even the most dedicated practitioners.

We do a lot of troubleshooting for our customers. Because we are one of the leading suppliers of fixatives other than neutral buffered formalin (NBF), we field many especially challenging questions. One particularly intriguing and recurring problem is why some labs are able to use certain antibodies successfully with alternative fixatives while others are not. One group even published a letter pointing out the difficulty of performing IHC with specimens fixed in Prefer¹, using Ki-67 (MM1) and bcl-2 as examples. In their study, hyperplastic tonsils fixed in Prefer or NBF were embedded together and treated to a routine (but unspecified) heat-induced epitope retrieval (HIER) process suited for formalin-fixed tissue before staining. Not surprisingly, the formalin-fixed tissue gave better results, given that the protocol was optimized for formaldehyde fixation. We easily confirmed their results using buffers in the standard pH ranges. However, we succeeded in reliably producing fine staining in Prefer-fixed specimens (Figs. 1a-b) simply by optimizing the process to favor glyoxal fixation (see sidebar, page 2). Similar results were achieved with bcl-2 (Figs. 10a-b).

In a similar situation, an antibody vendor recommended against this fixative for its EGFR kit², which includes a protease digestion step to unmask the epitope. Prefer-fixed tissue fares poorly under those conditions because the enzyme damages the specimen rather severely. This protocol again has been optimized for formaldehyde fixation and cannot be expected to work with equal success with other fixatives. Here the solution was even simpler than with Ki-67 and bcl-2: we just omitted the enzyme and got picture-perfect results (Fig. 13).

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Yes, it can be done!

Demonstration of Ki-67 (Mib1) in Prefer-fixed tonsil, achieved with a simple, glyoxal-specific antigen retrieval procedure.

Fig 1a. Tonsil. 20x  
Fig 1b. Adenocarcinoma of the colon. 20x
We began asking labs if they were encountering similar failures with glyoxal-fixed specimens. Some did, many did not. It struck us as incongruous that our experiences and those of many of our customers differed so drastically from those who reported difficulty, so we initiated a research program to answer several questions:

1. Could we get the tests to fail?
2. Could we get the tests to work properly?
3. Could we develop successful protocols?
4. Is there a rational scientific explanation for our findings, and if so, what is it?

We are pleased to announce that all of these questions have been answered with a resounding “Yes”. Of greater importance and wider interest is the body of general knowledge that we obtained in the course of our research. There are principals, guidelines and advice that apply to any immunohistochemical operation regardless of the fixative used. Because of the universal value of these ideas, we have assembled them together as a booklet titled, *Lessons in Immunohistochemistry*. This is available free to anyone upon request.

We don’t claim to have originated many of these ideas, but at least we are putting them down in one place in logical order.

*Lessons in Immunohistochemistry* will help you interpret negative results, understand exactly how fixation influences IHC, and appreciate how and why antigen retrieval works. The booklet also stresses standardization whenever practical, and optimization of procedures at all times. Together, these lessons will give you real control over your staining results.

The rest of this issue of *The Innovator* contains specific, practical tips for labs using glyoxal-based fixatives like *Prefer*, or facilities receiving specimens so fixed. We are deeply grateful for the considerable support given to us by Vision Biosystems (Norwell, MA). Their Bond™-X immunostainer afforded sufficient control over the staining process that we were able to identify otherwise hidden sources of variability in critical comparisons. With few exceptions, we used their detection system and primary antibodies as well.

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**Antigen retrieval for glyoxal-fixed specimens**

1. Mount sections on positively charged slides (or other effective product).
2. Deparaffinize and hydrate slides to water.
3. Use either a Decloaking Chamber (Biocare Medical, Walnut Creek, CA) or a microwave pressure cooker.

**For the Decloaking Chamber:**
- Fill with room temperature water, place slides into appropriate containers filled with our buffer Exposé, or make your own 0.01 M tris buffer at pH 8.6.
- Program to run for 10 minutes at 125° C with a cooldown time of 10 minutes.
- Remove slides immediately and rinse in room temperature water.
- Proceed with immunohistochemical staining.

**For the microwave pressure cooker:**
- Fill device with our buffer Exposé, or make your own 0.01 M tris buffer at pH 8.6.
- Place slides into an appropriate slide rack.
- Seal the pressure cooker and place it into a microwave oven.
- Apply heat at full power.
- When the weight over the valve starts to rock, continue heating for 10 minutes.
- Remove device from the oven and allow to cool 10 minutes.
- Carefully remove the weight from the valve to release pressure.
- Open the lid and remove slides immediately.
- Rinse in room temperature water.
- Proceed with immunohistochemical staining.
We started our research by taking antibodies that were presenting difficulties. Whenever we could, we obtained detailed protocols from reporting facilities. We did our best to duplicate those conditions to confirm failure of staining, then tried various alternative procedures. *It was immediately apparent that the overwhelming cause of failure was performing antigen retrieval.* Simply omitting that step led to successful staining for many problematic antibodies. Doing it when it should not be performed destroyed immunoreactivity and sometimes compromised morphological structure. *The second most common cause of failure was using an inappropriate means of antigen retrieval when epitopes did need to be unmasked.*

If you are having trouble getting an antibody to work on glyoxal-fixed tissue, first try omitting antigen retrieval. If that doesn’t work, a special HIER procedure is warranted. Techniques for unmasking formalin-fixed antigens rarely work with glyoxal. Our procedure (see sidebar) gives consistent results, is easy to perform, and involves a single buffer solution and heating protocol.

Table 1 (on page 6) lists the antibodies we have tested to date. We identify the clones and sources that were used; others may be equally satisfactory, but you should verify that in your lab. All HIER was performed in the Decloaking Chamber and duplicated in the microwave pressure cooker, as detailed in the sidebar. All staining was accomplished on Vision Biosystems’s Bond™-X stainer using Bond™-X Polymer DS 9958 detection system, except for EGFR which used DakoCytomation’s pharmDX™ K1492. If you use other detection methods, you may need to adjust time or antibody concentration.

As indicated in Table 1, quite a few antigens can be demonstrated nicely without antigen retrieval (Figs. 2-7). Many of these will be compromised with standard retrieval methods common to formalin-fixed tissues.
A few antibodies seem to work equally well with or without antigen retrieval (Figs. 8-9). For convenience these can be batch-processed with others requiring HIER. In a related category, some antibodies like those for progesterone receptor, might need antigen retrieval when the antigen is not heavily expressed, as in normal tissue.

Finally, there are those antigens that become unreactive after glyoxal fixation, many of which are associated with nuclear histones. Glyoxal forms a cyclic compound with arginine, a major constituent of histones. This may block recognition of the epitope by the antibodies. Special high temperature, high pH retrieval permits good staining without compromising morphology. Particularly troublesome Ki-67 and bcl-2 are readily stained with this procedure (Figs. 10a-b).

Figure 5a.
Demonstration of cytokeratin 5 with CK5 antibodies, without the use of antigen retrieval. Omental mesothelioma, Prefer. 10x.

Figure 5b.
Myoepithelial cells are vividly demonstrated with CK5 antibodies, without the use of antigen retrieval. Prostate, Prefer. 20x.

Figure 6.
Cytokeratin 7 within duct epithelium is specifically stained here with CK7 antibodies, without the use of antigen retrieval. Ductal adenocarcinoma of the breast, Prefer. 20x.
While most of our examples involving antigen retrieval were prepared using the Decloaking Chamber, equally satisfactory results were obtained with a microwave pressure cooker set with equivalent parameters (Fig. 11).
Table 1.
Recommended procedures for IHC on glyoxal-fixed tissue

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Source¹</th>
<th>Dilution</th>
<th>Time (min)</th>
<th>HIER²</th>
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<tr>
<td><strong>Antibodies that should not be used with antigen retrieval</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD10 [Fig. 2]</td>
<td>58C6</td>
<td>NCL-L-CD10-270</td>
<td>1:100</td>
<td>20</td>
<td>No</td>
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<td>CD20 [Fig. 3]</td>
<td>L26</td>
<td>NCL-L-CD20-L26</td>
<td>1:800</td>
<td>8</td>
<td>No</td>
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<tr>
<td>CD117 [Fig. 4]</td>
<td>T5B5</td>
<td>NCL-L-CD117</td>
<td>1:40</td>
<td>20</td>
<td>No</td>
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<tr>
<td>Chromogranin</td>
<td>5H7</td>
<td>NCL-CHROM-430</td>
<td>1:100</td>
<td>8</td>
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<tr>
<td>CK5 [Figs. 5a,b]</td>
<td>XM26</td>
<td>NCL-L-CK5</td>
<td>1:500</td>
<td>20</td>
<td>No</td>
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<tr>
<td>CK6³</td>
<td>LHK6B</td>
<td>NCL-CK6</td>
<td>1:400</td>
<td>20</td>
<td>No</td>
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<td>CK7 [Fig. 6]</td>
<td>LP5K</td>
<td>NCL-CK7</td>
<td>1:10</td>
<td>20</td>
<td>No</td>
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<tr>
<td>CK20⁴</td>
<td>543</td>
<td>NCL-CK20</td>
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<td>8</td>
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<tr>
<td>CK HMW (1/5/10/14)⁵</td>
<td>34BE12</td>
<td>NCL-CK34BE12</td>
<td>1:200</td>
<td>20</td>
<td>No</td>
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<tr>
<td>Desmin [Fig. 7]</td>
<td>DE-R-11</td>
<td>NCL-L-DES-DER11</td>
<td>1:100</td>
<td>20</td>
<td>No</td>
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<td>EGFR [Fig. 13]</td>
<td>2-18C9</td>
<td>Dako K1492</td>
<td>per kit</td>
<td>30</td>
<td>No</td>
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<td><strong>Antibodies that work equally well with or without antigen retrieval</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD31</td>
<td>1A10</td>
<td>NCL-CD31-1A10</td>
<td>1:100</td>
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<tr>
<td>HMB45 [Fig. 8]</td>
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<td>NCL-HMB45</td>
<td>1:40</td>
<td>20</td>
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<td>Vimentin [Fig. 9]</td>
<td>VIM 3B4</td>
<td>NCL-VIM</td>
<td>1:100</td>
<td>8</td>
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<td><strong>Antibodies that might need antigen retrieval for normal controls</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PR</td>
<td>16</td>
<td>NCL-PGR-312</td>
<td>1:300</td>
<td>20</td>
<td>No/Yes</td>
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<td><strong>Antibodies that must have antigen retrieval</strong></td>
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<td></td>
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<td>bcl-2 [Fig. 10a,b]</td>
<td>100/D5</td>
<td>NCL-L-bcl-2</td>
<td>1:100</td>
<td>20</td>
<td>Yes</td>
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<td>c-erb-2 [Fig. 12]</td>
<td>CB11</td>
<td>NCL-CB11</td>
<td>1:100</td>
<td>20</td>
<td>Yes</td>
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<tr>
<td>ER [Fig. 11]</td>
<td>6F11</td>
<td>NCL-ER-6F11</td>
<td>1:120</td>
<td>20</td>
<td>Yes</td>
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<tr>
<td>KI-67⁶ [Fig. 1a,b]</td>
<td>Mib-1</td>
<td>Dako M-7240</td>
<td>1:150</td>
<td>20</td>
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<td></td>
<td>BX297</td>
<td>Biogenex</td>
<td>1:30</td>
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<tr>
<td>DVB-2</td>
<td>Biocare CM080B</td>
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<td>MM1</td>
<td>NCL-i-Ki67-MM1</td>
<td>1:50</td>
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<td></td>
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<tr>
<td>K2</td>
<td>Ventana 790-2910</td>
<td>per kit</td>
<td>20³</td>
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<tr>
<td>TTF-1⁹</td>
<td>SPT24</td>
<td>NCL-TTF-1</td>
<td>1:150</td>
<td>20</td>
<td>Yes</td>
</tr>
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</table>

¹ NCL = Novacastra Laboratories
² No = no pretreatment is best, HIER produced tissue damage or diminished staining
   Yes = HIER is necessary
   Optional = no pretreatment or HIER equally satisfactory on all tissues
   No/Yes = no pretreatment is adequate for richly positive tissues but normal controls do better with HIER
³ See Fig. 15b in Lessons in Immunohistochemistry.
⁴ See Fig. 8 in Lessons in Immunohistochemistry.
⁵ See Fig. 7 in Lessons in Immunohistochemistry.
⁶ See Figs. 11c,d in Lessons in Immunohistochemistry.
⁷ Slight background staining suggests reducing time in primary antibody.
⁸ See Fig 9b in Lessons in Immunohistochemistry.
A note on the use of Prefer-fixed tissue for assays involving EGFR and HER-2

The last item in our booklet, Lessons in Immunohistochemistry, addresses the need to validate any antibody being introduced into your lab. Please read that carefully. It provides the key to performing assays for antigens tied to therapeutic treatment.

To qualify for treatment with Herceptin or Erbitux, the patient must be tested to verify that their tumor cells are expressing the HER-2 or HER-1 protein, respectively. This is accomplished histologically by using the corresponding antibodies with validated procedures. FDA-approved kits are available, but are not the only option open to you and they do not eliminate the need to validate them in your own lab. These kits prescribe stringent conditions of fixation which appear to preclude the use of alternative fixatives or fixation times other than those specified.

FDA-approved antibodies can be used with divergent protocols, just as other suitable brands of appropriate antibodies can be used. Any deviations from the package inserts must be validated within your facility. The package inserts for the drugs (not the diagnostic antibodies) describe the method of validation. In essence, it involves performing assays using your procedures on your specimens previously diagnosed to see if your methods result in the correct diagnosis in an acceptable percentage of cases. This is exactly the method used by antibody companies to win FDA approval of their product. The Clinical and Laboratory Standards Institute (CLSI, formerly known as NCCLS) provides another way to perform validation: perform assays on 15-20 cases in which the diagnosis is well established, and another 15-20 cases likely to be histologically confused.

That might seem to involve a lot of work, but it is not as burdensome as it sounds. Furthermore, you should be doing it anyway even if you use the kits as prescribed, if you wish to be in compliance with CLSI guidelines. Different labs are never identical no matter how many constraints are placed on operational methods, hence the need for local validation of all antibodies. Using Prefer simply means that your specimens will be fixed differently and perhaps stained with an altered protocol; it does not mean you have to do any more than you would if NBF was your fixative.

Our recommendations for these two assays are simple. For HER-1, just omit the step involving antigen retrieval to prevent destruction of immunoreactivity (Fig. 12). With HER-2, our recommended HIER procedure at pH 8.6 is necessary (Fig. 13).

Figure 12.
Successful demonstration of HER2. Ductal adenocarcinoma of the breast, Prefer. HIER at pH 8.6, c-erb-2. 20x.

Figure 13.
Successful demonstration of EGFR. Colonic adenocarcinoma, Prefer. DakoCytomation’s EGFR pharmDX™ kit without prescribed antigen retrieval. 20x.
Exposé, a new way to retrieve antigenicity

To date, all antibodies tested have performed well on Prefer-fixed tissue. When retrieval was necessary, a single procedure sufficed: treatment in a microwave pressure cooker or a Decloaking Chamber at 125° C with a pH 8.6 buffer. You may prepare your own buffer, using 0.01 M Tris HCl, but a more convenient way is to purchase our specially formulated solution, Exposé. It contains a preservative free of mercury and is carefully manufactured so that you will have reproducible results. The pH is critical. Our studies have shown that if it is lower, staining is impaired or abolished. If the pH is above 9.0, tissue damage is likely. [Note: pH values cited here refer to readings at room temperature]. Exposé is your key to better IHC when Prefer or other glyoxal fixatives are involved.

Catalog #601 ........... Exposé ............. 2x1 qt case

We are ready to help

We conclude this issue of The Innovator with an offer of assistance to anyone having difficulty performing immunohistochemistry on tissue fixed in Prefer. We will work out staining protocols in our lab at no charge, provided you send us suitable control and/or pathological tissue. We also need complete details on how the specimens were fixed and processed. Caution: tissues fixed in formalin then Prefer, or Prefer then formalin, may be irretrievably blocked, but we will do our best to effect satisfactory results. Please contact us for details before sending specimens.

Additionally, we are in contact with reference labs which are eager to perform IHC on Prefer-fixed tissue. If you need such service, please call us for referrals. We are not affiliated with any labs, and make referrals only because of a common professional interest in providing highest quality health care.

References


2 DakoCytomation package insert for EGFR pharmDX™ Kit

3 Clinical and Laboratory Standards Institute (formerly NCCLS), 1999. Quality Assurance for Immunocytochemistry; Approved Guideline. CLSI Document MM4-A: Section 8.6, page 39

Call for free booklet!

(See inside this Innovator for more information.)