WHITEPAPER

Biocare Basics: Optimization

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Proper optimization is vital for successful immunohistochemical (IHC) testing. An optimal IHC protocol can be characterized by its ability to consistently and unambiguously determine how the antigen is expressed in the tissue. Optimization is fundamental to reducing the risk of both false positive and false negative results.[1] The protocol must be able to identify both high and low expression of target antigens in normal tissue while also identifying unknown levels of expression in abnormal tissue.[1]

There are two approaches for optimizing a new antibody protocol. The first is most likely to be used by researchers when developing new "homegrown" antibodies, in which case no previous documentation exists.[2] For this approach, the researcher must create a new protocol and test all variables one at a time before delineating results. The second, more common approach is suggested for clinical applications. It involves researching the antibody and planning a protocol using the wealth of preexisting information available.[2] Resources available for review include the antibody or detection vendor's website or catalog, reagent specification sheets, journal articles, and textbooks. These resources can provide a basic understanding of the antibody and may recommend retrieval methods, the antibody dilution range, the type of detection to use, and positive and negative controls. In addition, knowing expected labeling patterns and cellular location of expression, such as nuclear or cytoplasmic, can assist when reviewing results.

An example of a standard IHC protocol is as follows: antigen retrieval, blocking steps (peroxidase or endogenous protein), primary antibody application and incubation, visualization through the detection system and chromogen, and, lastly, counterstain. Since the reagents, time, and temperature of each step can vary greatly, it is critical to only change one variable at a time when optimizing.

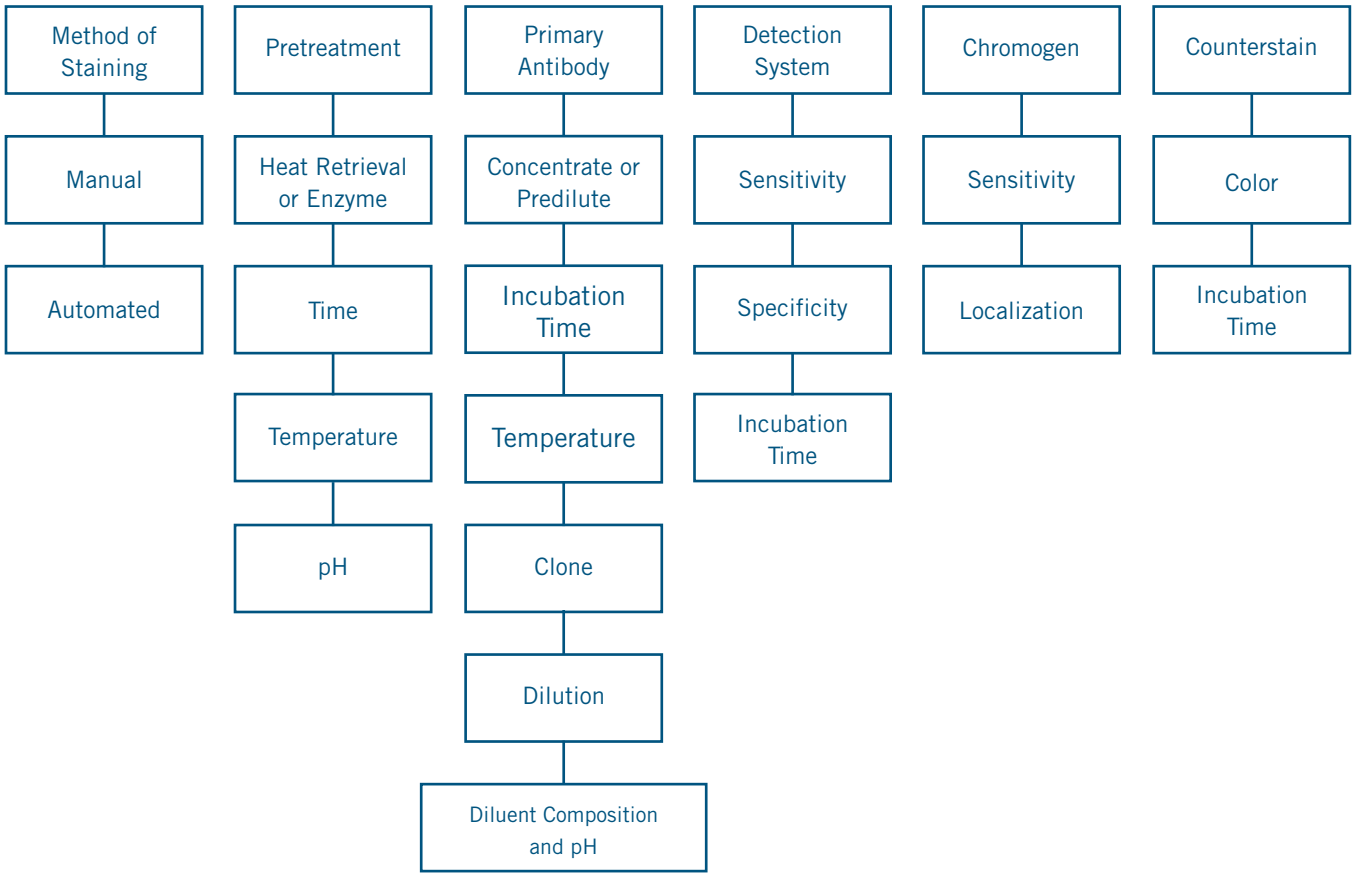
Some labs may start with testing the retrieval conditions by varying the pH and composition at different times and temperatures or testing an enzyme digestion. It is recommended that when an enzyme is introduced, times of 5, 10, 20, and 30 minutes in the enzyme solution be evaluated to ensure optimal digestion is achieved and that the tissue is not over-digested.[2] It is important to note that some antibodies do not require any retrieval, and a retrieval step may introduce false-positive staining.

Upon receiving a new concentrated antibody, a lab will typically perform serial dilutions of the antibody (1:100, 1:200, 1:400, and 1:800) using a known positive and negative tissue control. The manufacturer will typically indicate an approximate dilution; however, several different dilutions should be tested with the standard laboratory protocol to determine the correct dilution for your lab. The optimal dilution will present the best signal-to-noise ratio, meaning a crisp stain with the strongest possible intensity without generating background. When diluting an antibody, it is important to use a diluent that contains carrier proteins and the proper pH for antibody stability.[2] (For more information, see Biocare's whitepaper entitled "Differentiating Diluents: Tricks of the Trade for Diluting Antibodies). On the other hand, ready-to-use (RTU) antibodies are "prediluted," meaning the vendor has previously established the optimal dilution of the antibody and diluent.

Before selecting a visualization system for the antibody, the antibody application and tissue type should be considered. For instance, brown-colored DAB may be avoided when visualizing antibodies on dermatological specimens to prevent masking by brown melanin pigment. Lastly, counterstain must be optimized to reduce variability in its intensity. A too intense counterstain may lead to optical distortion (for nuclear antigens in particular), and too weak can affect the tissue's morphological assessment.[1]

As tumors are known to exhibit very heterogeneous antigen expression, it is crucial that your IHC protocol is optimized to detect varying levels of expression.[1] Once the appropriate protocol is determined for a given antibody, multiple positive and negative tissues should be tested to ensure the targeted cells are labeling appropriately. This testing will validate sensitivity, specificity, accuracy, and reproducibility before use on patient tissue.[2] A record of all tested parameters, including the optimized protocol and results, should be maintained for each antibody, along with proof of validation when completed.

Conventional IHC Procedure Steps and Corresponding Variables that May Affect Staining



Common Optimization Troubleshooting Parameters[3]

Insufficient Staining Observed	Excessive Non-Specific Staining Observed
Extend pretreatment time or increase temperature	Decrease pretreatment time or decrease temperature
If using HIER, test an Antigen Retrieval Solution with a higher pH	If using HIER, test an Antigen Retrieval Solution with a lower pH
Increase antibody concentration	Include an endogenous protein and peroxidase block step
Increase antibody incubation time	Decrease antibody concentration
Increase incubation time of secondary antibody (also known as “linker”, “probe”, or “post primary”)	Decrease incubation time of secondary antibody (also known as “linker”, “probe”, or “post primary”)
Increase incubation time of tertiary antibody (also known as “polymer”)	Decrease incubation time of tertiary antibody (also known as “polymer”)

Need assistance optimizing your antibody protocols? We are here to help!

Biocare Medical’s team of certified Field Applications Specialists can provide antibody optimization services on all Biocare reagents and instrumentation. Our Applications team remains instrument agnostic- offering IHC optimization services on any and all IHC platforms. Have questions on available services? Please contact your local Sales Representative or call 1-800-799-9499.

1. Jacobsen, L., Nielsen, M., Mansson, S., Rudbeck, L. (2013). Staining Protocol Optimization. In C. R. Taylor & L. Rudbeck (Eds.), Immunohistochemical Staining Methods (6th ed., pp. 61-77). Dako Denmark A/S.

2. Carson, F. L., & Cappellano, C. H. (2015). Histotechnology: A Self Instructional Text (4th ed.). Chicago, Illinois: ASCP Press.

3. Myers, J. Conceptualization of Immuno-Staining Procedure Optimization. 12 August 2020.