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Immunohistochemistry

Optimal Titering of Primary Antibodies
(And the Dangerous Fallacy of "Predilute Ready-to-Use" Antibodies)

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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 at the appropriate titer is very important. A crucial point to know 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 blind acceptance of this fallacy is responsible for many problems in both neophyte and established laboratories. A related point is to **NEVER BELIEVE THE MANUFACTURER'S RECOMMENDATIONS FOR TITER OF THE PRIMARY ANTIBODY**. Sometimes they will be correct in their suggestions but, in other situations, the titer that they 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 used 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.

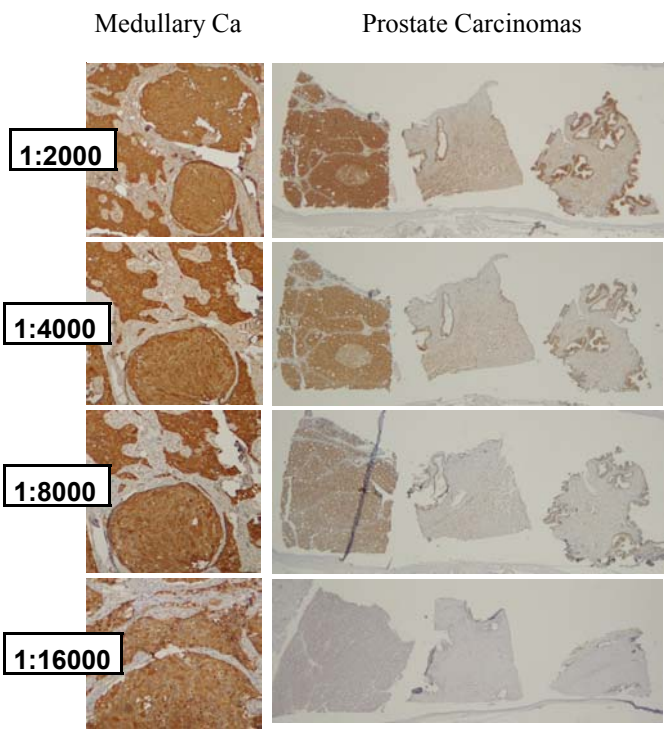
Titering of antibodies can be one of the most challenging aspects of immunostaining for people just learning the technique, and I believe that the failure to determine optimal titers is the main reason for frustrating results (or perpetual mediocrity) experienced by many laboratories. Determining optimal titers is not difficult, but it involves some work as well as awareness of the features of a "true positive" and a "false positive" immunostain (please see the December 2001 "Focus on Immunohistochemistry" for a discussion of true positive and false positive stains).

The first step in determining optimal titers is to decide on a series of titers to try. If working with a new antibody, it is usually best first to determine whether any pretreatment is needed and, if so, which epitope retrieval method (protease digestion or heat-induced retrieval in citrate buffer, Tris buffer, EDTA, etc., in a pressure cooker, steamer, or microwave) 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, and 1:800, (using the optimal method of epitope retrieval that has been previously determined). Usually, but not always, an appropriate titer can be chosen from the first set of studies and you 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 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, you 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. This may seem logical at first glance, 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 is obviously important to as-

sure 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 expected negative cases are indeed nonreactive for the antigen in question.**

Calcitonin Antibody Titrations



Series of polyclonal calcitonin antibody titers illustrating the importance of proper titration studies employing multitumor blocks. The left column consists of calcitonin immunostains performed on medullary thyroid carcinoma at the corresponding primary antibody titers listed at the left. The right column illustrates 3 prostate adenocarcinomas present on the multitumor sandwich block sections (present on the same slides) that were used for the titration studies. Note that appropriate staining is achieved only when the antibody is diluted to 1:16,000, since inappropriate staining of the prostate carcinomas is present at more concentrated titers. This highlights the importance of evaluating expected positive tumors and expected negative tumors when determining optimal titers. In this case, the manufacturer-suggested titer (which would have been sold as "ready-to-use") of 1:500, way off the mark.

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 a few laboratories 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 titering antibodies (a modification of Battifora's original "sausage block" and "checkerboard" technique, and the conceptual forerunner of currently popular tissue microarrays). 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.

REFERENCE: Miller, RT: Multitumor "Sandwich" Blocks in Immunohistochemistry. Simplified Method of Preparation and Practical Uses. *Applied Immunohistochemistry 1(2):156-159, 1993.*

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