What You Should Know (and READ) Before Beginning Immunostaining

(According to Drs. W. Grizzle, et. al.)

Immunohistochemical Staining (Bright Field)

A. Preparation of Paraffin Sections for Immunohistochemistry: For most tissues, paraffin sections are cut at 5 μM, mounted on sialinized slides (Plus slides), and melted at 65^{o}C in an oven for 2h to aid in attachment of sections to glass slides. It has been demonstrated that most antigens are stable with respect to immunorecognition when slides are melted at less than 70^{o}C for up to 24h [1]. In tissues with a lot of fat (e.g., breast) it may be necessary to attach the sections for longer than 2 hours to keep the samples from detaching, especially during antigen retrieval approaches.

B. Antigen Recovery Techniques: There are several important issues with using any type of antigen retrieval/recovery. First, most forms of antigen recovery affect the sensitivity of the immunohistochemical assay, but such effects vary with a) the tissue, b) the antigen, c) the antibody and d) the conditions of antigen recovery (primarily time, temperature and pH). Antigen recovery may unmask specific forms of staining that are reduced by fixation and hence, may not be detected upon staining paraffin sections that have not undergone antigen retrieval procedures. This is especially the case in some tissues (e.g., kidney) in which there is a high biotin activity. This will be a problem for if some form of avidin binding is being used in the detection of the primary antibody. Also, antigen recovery may induce other staining artifacts. Thus, for any type of antigen recovery (AR) performed, a matching "control" slide in which the primary antibody has been deleted (e.g., the AR delete) must be performed in order to identify such problems. The exact form of antigen recovery must be matched in the AR delete and if several forms of AR are tested on a tissue, an AR delete must be evaluated for each type of AR.

For some antigens, such as p53, experience indicates that antigen retrieval techniques may be too sensitive and confuse correlations of nuclear accumulation of p53 with clinical outcome [7, 8]. In contrast, for some tissues

such as colorectal adenocarcinoma and some antigen-antibody combinations (e.g., Bcl-2), a method of antigen retrieval may be necessary to identify the expression of the antigen. (The effect of AR on the clinical question must be determined.) The decrease of immunorecognition of fixation and paraffin processing varies with the technique and type of fixation [9] as does the antigen recovery necessary to reverse fixation/processing effects. Some antigens (e.g., Ki67/MIB-1) cannot be recognized in sections from paraffin processed blocks unless antigen retrieval techniques are used. Also, the method of antigen retrieval varies with the antigen-antibody combination. For Bcl-2, the following technique may be used: The sections on Plus® slides are placed in citrate buffer at pH = 6. The sections are then boiled using a microwave oven for 5 minutes, the volume of buffer is replenished with hot buffer (> 90°C), and then the citrate buffer is boiled for an additional 5 minutes.

For some nuclear antigens, e.g., ER and Ki67/MIB, it has been reported that trypsin treatment followed by 2hr of 80°C, pH6.0 citrate incubation (low temperature antigen retrieval) gives excellent results [10], while other antigens – antibody combinations give best results in pH8.0 EDTA solution heated for 30 minutes in a pressure cooker. Currently, the pH = 8.0 EDTA pressure cooker method is preferred for most antigens.

C. Detection of Antigens in Paraffin Sections: Prior to adding primarily antibody, goat serum is added to the tissue section in order to block non-specific immunostaining. The sections are then ready to be incubated with the primary antibody being studied. The primary antibody is added at a dilution (made in Tris buffer) selected as described [2, 3] and the section is then exposed to the primary antibody. For each antigen-antibody combination, the time of incubation and the temperature of incubation may vary as does whether or not antigen retrieval techniques are used.

D. Selecting the Proper Conditions for Immunostaining: Before establishing the conditions of immunostaining, perform a Western blot using extract of tissue of interest and antibody to be optimized. Identify all bands on Western to ensure specificity. For antigens with which one has no experience, as will probably be the case for many of the antigens identified in EDRN studies, one should optimize the conditions of immunostaining. Because it is convenient to keep the conditions of secondary detection relatively constant, the first steps are to optimize the concentrations of the primary antibodies and the conditions of antigen retrieval (recovery). This usually requires one to test various

concentrations of the primary antibody after the conditions of antigen retrieval have been optimized. First, one can start at a low (1µg/ml), medium (5µg/ml), and high (10µg/ml) concentrations of primary antibody and the condition recommended by the source of the antibody using no form of antigen recovery and test typically 5 antigen retrieval conditions. It is preferable to know the immunoglobulin concentration (protein concentration) of any antibody used in immunohistochemistry or immunoassays in general; however many sources of antibodies provide only a recommended dilution. One should attempt to obtain the exact antibody concentration from the supplier but this is frequently not possible. This concentration must be determined by the source, e.g., preferably after affinity purification and before the addition of any proteins (albumin) added to stabilize the freezing of the antibody. If the concentration cannot be / is not provided, then one must work with dilutions. Generally the upper limit of a concentration of a primary antibody is 20 µg/ml; this concentration usually is too concentrated for staining and thus, all the tissue will be stained without differentiated staining. For a polyclonal antibody obtained from blood, a serum dilution of 1/20 also is usually limiting with generalized staining with a differential pattern representing the antigen of interest. When the antigen retrieval conditions are optimized, then one can optimize the concentration of the primary antibody.

The optimized concentration is determined by staining using a standard secondary detection system (e.g., Signet). The time of exposure to the secondary detection system should **not** be less than 10 minutes to avoid overstaining and to reduce errors in timing. Upon examining the immunostaining with the low and high concentrations of primary antibody and no AR take the following steps:

1) If no staining is observed, move to a 15µg/ml concentration or a 1:20 dilution. If no staining is observed, go to maximum antigen retrieval/recovery protocol. If no staining is observed, either the antibody cannot be used for immunohistochemistry or the protein concentration of the antibody is incorrect

2) If strong staining is observed at 1µg/ml, continue to stain at 500, 100, 10, 1 and 0.1 ng/ml or until staining intensity is reduced. When a decrease in staining is observed (e.g., average 1.5 of 4.0 max – see section on evaluation), go to maximizing antigen retrieval/recovery protocol repeating #1) and #2) above..

3) If staining is observed at 5 µg/ml, increase or decrease the antibody concentration until an average staining of 1.5 of 4 is observed.

E. Secondary Detection of the Primary Antibody: Current secondary detection methods for monoclonal antibodies may use an anti-mouse, anti-rabbit detection system from Signet (Multi-Species Ultra Streptavidin Detection System HRP®, Signet Laboratories, Inc., Dedham, MA). This detection system was chosen because of the reproducibility of various lots compared to other vendors. Signet works with specific laboratories to ensure standardization.

F. Counterstaining is needed in most cases—see specific SOP.

G. Evaluation and Analysis of Immunohistochemistry: The method used for evaluating immunohistochemistry was developed in the laboratory of Dr. William E. Grizzle [1, 2]. The specific immunohistochemical techniques used in his laboratory have been described in detail (reviewed in [1, 2, 8, 11]. Once immunohistochemistry has been completed, then an important issue arises as to how the immunohistochemistry is to be evaluated. Dr. Grizzle's method for evaluating immunohistochemistry is described as follows. The tumor cells or pre-invasive neoplastic cells to be evaluated are selected by a point counting technique. Specifically a grid is used on photographs to randomly select tumor cells for evaluation (those cells that fall on the intersection of grid lines are selected). This is a standard technique of stereological analysis. Each cell is evaluated separately for membranous, cytoplasmic and nuclear staining. For cytoplasmic and membranous staining, the tumor cells are classified with respect to the intensity of immunostaining for each antigen with the percent of cells determined at each staining intensity from 0 to +4. It should be emphasized that the stoichiometry of the reaction between an antigen and the ultimate colored precipitate which is deposited at the antigen location by the primary antibody detection system is not one to one. Specifically one molecule of substrate does not interact for each molecule of antigen identified by the primary antibody. Thus, immunohistochemistry using the supersensitive detection techniques currently available produces results which are not linear. Since the stoichiometry is unknown, one can not conclude that an intensity of +3 is 3 times the expression of protein identified by an intensity of +1; however, one can demonstrate using sequential antibody dilutions

that an intensity of +3 indicates more antigen is present than an intensity of +2 or less. Therefore, efforts at performing analysis with extreme accuracy are probably not warranted and it is controversial as to exact results using semiquantitative immunohistochemistry; however, this semiquantitative method has been reported to mirror biology and to correlate with other more quantitative methods (protein chemistry and enzymatic chemistry). Specifically, using cell lines transfected with the enzyme, cytosine deaminase (CD), which is not expressed in human cells, it has been demonstrated that this method of evaluation correlates with 1) the MOI of transfection, 2) the protein levels of CD in cells, 3) enzymatic activity of CD in cells and 4) cell killing by addition of 5-flurocytosine.

Table 1: Reproducibility of Staining with the Antibody CC-49 (TAG-72)

Tissue	Antibody CC-49 concentration µg/mL	Repeat measurements R_1	R_2	R_3	R_4	Average	SD
Spleen white pulp	0.1	0	0	0	0	0	0
Spleen white pulp	1.0	0	0	0	0	0	0
Spleen white pulp	10.0	0	0	0	0	0	0
Colon tumor 1	0.1	1.8	1.7	1.55	1.7	1.69	0.10
Colon tumor 1	1.0	2.1	1.4	1.7	1.6	1.70	0.29
Colon tumor 1	10.0	2.5	2.0	2.4	2.6	2.37	0.26
Smooth muscle In colonic wall 1	0.1	0	0	0	0.3	0.07	0.15
Smooth muscle In colonic wall 1	1.0	0	0	0	0	0	0
Smooth muscle In colonic wall 1	10.0	0.1	0	0.3	0	0.10	0.14
Colon tumor 2	0.1	3.2	1.6	1.55	2.9	2.31	0.86
Colon tumor 2	1.0	3.2	2.3	2.6	2.9	2.67	0.51
Colon tumor 2	10.0	3.2	2.0	2.4	3.0	2.65	0.55
Smooth muscle In colonic wall 2	0.1	0	0	0	0	0	0
Smooth muscle In colonic wall 2	1.0	0	0.5	0	0	0.12	0.25
Smooth muscle In colonic wall 2	10.0	0	0.5	0.7	0	0.30	0.36

[a]Each repeat measurement (e.g., R_1, R_2, R_3, and R_4) represents a different staining run and separate grading (blinded) by the same observer.

This semiquantitative method also correlates with real time quantitative PCR analysis of mRNA for CD [12]. Also, this method of analysis has been found to correlate with clinical outcomes for multiple molecular markers [1-3, 7-12]. One would like assay results to be as consistent as possible so that the standard error of repeat assays can be reduced and hence statistical changes in stage-specific expression of biomarkers in neoplasia can be evaluated. To permit numerical analysis the proportion of cells at each

intensity can be multiplied by that intensity. A score can be developed that ranges from 0 to 4 (see examples below):
To permit numerical analysis the proportion of cells at each intensity can be multiplied by that intensity. A score can be developed that ranges from 0 to 4 (maximum). E.g., consider the tumor staining as follows for TGFα.

INTENSITY	0	1	2	3	4	
% CELLS STAINING	40	20	30	10	0	**TOTAL SCORE**
SCORE	0	0.2	0.6	0.3	0	1.1

This would be classified as relatively weakly staining with a total score of 1.1

In contrast, a tumor staining as follows for $p185^{erbB-2}$.

INTENSITY	0	1	2	3	4	
% CELLS STAINING	0	10	20	40	30	**TOTAL SCORE**
SCORE	0	0.1	0.4	1.2	1.2	2.9

This would be classified as staining strongly with a total score of 2.9. We have used this semi-quantitative approach with various models of immunohistochemical staining of antigens. Our models have indicated that this method of evaluation is less sensitive to variation in the intensity of staining than other methods of evaluation (see appendix, manuscripts Miller and Poczatek).

This semi-quantitative approach has been used with various models of immunohistochemical staining of antigens. Models have indicated that this method of evaluation is less sensitive to variation in the intensity of staining than other methods of evaluation. In addition, Table 1 demonstrates the ability of this method to yield consistent immunohistochemical assays and to grade assays consistently. It also demonstrates the importance of selecting the correct conditions for immunoassays and the problems that may occur in overstaining.

H. Quality Assurance: The following approach is useful in the quality assurance for immunopathological assays. First, as a method of quality control, 10% of cases are selected randomly and all the immunoassays are repeated and compared with prior results; if there is a difference of more than

30% in any of the tests, the cause of the discrepancy is determined and corrected. This approach is an important part of a quality assurance program. One should practice not only quality control but also quality assurance for the assays performed in the laboratory. In addition to the method of quality control, all tests are performed with great care following standard operating procedure (SOP) described herein including appropriate controls. In each assay, at least one slide, the delete, is stained without application of the primary antibody. An antigen recovery (retrieval) delete is performed separately for each antigen recovery procedure that is performed in any assay. A multi-tissue control slide also is stained for each antigen. Several antigens known to be expressed in tissue under study are used as positive controls. These include nuclear antigens (e.g., $p27^{Kip-1}$, Ki67/MIB-1), and membrane antigens (EGFr and $p185^{erbB-2}$). All reagents are selected and purchased to ensure reproducibility of results. For example, one can buy a detection system in large bulk lots prepared especially for the laboratory. New lots of primary antibodies as well as detection systems are purchased so that a complete study can be completed with a specific lot. If studies extend for several years beyond the expiration date of reagents, new detection lots can be adjusted by the vendor (Signet) to meet the sensitivity of prior lots. Also, all assays are performed with great attention to all details of the procedures (e.g., time of staining).

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