BASIC IMMUNOCHEMISTRY

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■ Antibody titer and dilutions, as well as incubation time and temperature, are tightly interwoven in their effects on the quality of immunohistochemical staining. These factors can be changed independently or, as is more often the case, in complementary fashion, to bring about marked differences in the quality of staining. Generally, when making any changes, the overriding goal should be the achievement of optimal specific staining accompanied by minimal interference from background staining. This chapter will highlight these variables.

ANTIBODY TITER

■ In immunohistochemistry, the optimum antibody titer may be defined as the highest dilution of an antiserum (or monoclonal antibody) that results in maximum specific staining with the least amount of background under specific test conditions. This highest dilution is determined primarily by the absolute amount of specific antibodies present.

With polyclonal antisera, antibody levels have traditionally been expressed as micrograms of antigen precipitated per milliliter of antiserum. Although of interest, to the immunohistochemist this is not necessary information.

For monoclonal antibody preparations, the absolute concentration of specific antibodies can be readily measured and frequently forms the basis for making the required dilutions. The highest dilution is also governed by the intrinsic affinity of an antibody; if the titer is held constant, a high affinity antibody is likely to react faster with the tissue antigen and give more intense staining within the same incubation period than an antibody of low affinity.

Augmenting titers by isolating and enriching the immunoglobulin fractions from polyclonal antisera produces little benefit for immunohistochemical applications because nonspecific antibodies and soluble aggregates—frequently an additional source for nonspecific background—become enriched also (see Background chapter).

Titers as defined above may vary from 1:100 to 1:2000 for polyclonal antisera; from 1:10 to 1:1000 for monoclonal antibodies in cell culture supernatants; and up to 1:1,000,000 for monoclonal antibodies in ascites fluid. These dilutions may well be exceeded in the future by the ever increasing sensitivities of newer detection methods and, in some cases, by the use of an appropriate antigen retrieval procedure.

ANTIBODY DILUTION

■ Often the manufacturer offers prediluted reagents ready for use, or recommends dilution ranges compatible with other variables such as method, incubation time and temperature. If this information is not provided, determine optimal working dilutions of immunochemical reagents by titration. Correct dilutions will contribute to the quality of staining if they are prepared accurately and consistently. They are best determined by first selecting a fixed incubation time and then by making small volumes of a series of experimental dilutions. Depending on specimen size, applications of 0.1–0.4 mL of solution per section is generally adequate. On paraffin sections, optimal dilutions of primary antibodies are not only signaled by a peak in staining intensity, but also by the presence of minimal background (maximal signal-to-noise ratios). Once the optimal working dilution is determined, larger volumes can be prepared according to need and stability.

The extent to which monoclonal antibodies can be diluted is subject to additional criteria. Because of their more restricted pI and molecular conformation, monoclonal antibodies are more sensitive to the pH and ions of the diluent buffer.[1] It was proposed therefore that any evaluation of monoclonal antibodies also include their titration at pH 6.0 and 8.6 in the *absence* of NaCl. That highest dilution and pH retaining the strongest immunoreactivity was called the optimal dilution and recommended for future use. Of the diluents tested, phosphate buffered saline, although widely used as a diluent for primary antibodies, was found to suppress the reactivity of most monoclonal antibodies tested.

Dilutions are usually expressed as the ratio of the more concentrated stock solution to the total volume of the desired dilution. For example, a 1:10 dilution is made by mixing one part of the stock solution with nine parts of diluent. Two-fold serial dilutions are made by successive 1:2 dilutions of the previous stock dilution. In order to make a very small volume of a highly diluted solution, it may be necessary to make it in two steps. For example, to prepare 1.0 mL of a 1:1000 dilution, first make 100 µl of a 1:10 dilution (10 µl + 90 µl), and then 1000 µl of a 1:100 dilution using 10 µl of the first dilution (10 µl + 990 µl).

When making dilutions, the use of adjustable pipettes allows for greater flexibility and more accurate delivery. To measure volumes in excess of 1.0 mL, use serological or volumetric pipettes. Table 1 indicates the volumes of stock reagents and diluents necessary to obtain dilutions

ranging from 1:50 to 1:200. Checkerboard titrations are used to determine the optimal dilution of more than one reagent simultaneously. In this example of a checkerboard titration, the optimal dilutions of a primary antibody and the streptavidin-HRP reagent are to be found, while the dilution of the biotinylated link antibody is held constant (not shown). Nine tissue sections are required if three dilutions are to be tested.

TABLE 1

Streptavidin-HRP	Primary Antibody Dilutions		
1:50	1:50	1:100	1:200
1:100	1:50	1:100	1:200
1:200	1:50	1:100	1:200

As noted earlier, staining results achieved by use of several different dilutions will often be identical or similar, in which case the cost of the reagent may become an additional factor in selecting the optimal dilution.

Precise definition of the optimal signal-to-noise ratio as a function of the primary antibody dilution is likely to be more critical with some methods than with others. For example, it was found to be more restricted with the use of unlabelled enzyme-antienzyme complexes (PAP, APAAP), than with methods utilizing the (strept)avidin-biotin technology.[2] This is probably consistent with the observation that, as opposed to the PAP method, the ABC method cannot distinguish between high and low concentrations of tissue antigens.[3]

ANTIBODY INCUBATION

As mentioned previously, incubation time, temperature and antibody titers are interdependent; a change in one factor will affect the others.

INCUBATION TIME There is an inverse relationship between incubation time and antibody titer—the higher the antibody titer, the shorter the incubation time required for optimal results. In practice, however, it is expedient to first set a suitable incubation time before determining the optimal antibody dilution. Higher concentrations of specific antibodies (and higher affinities) allow for the shortening of the incubation time.

Incubation times for the primary antibody may vary up to 48 hours, with 10–30 minutes probably being the most widely used. For an antibody to react sufficiently strong with the bound antigen in a very short period of time, it must be of high affinity and of relatively high concentration. Variables believed to contribute to increased nonspecific background staining should be kept to a minimum (see Background chapter). Primary antibody incubations of 48-hour duration allow, more than anything else, for greater economy because very high dilutions of antiserum may be used. While antibodies of low affinity and/or low titer must be incubated for long periods in order to reach equilibrium*, nothing can be gained by prolonging primary antibody incubation beyond the time at which the tissue antigen is saturated with antibody.

Equilibrium is usually not reached during primary antibody incubations of less than 20 minutes. Consistent timing of the primary antibody incubation step is of great importance. Inconsistent incubation times can cause variations in the overall quality and intensity of staining. Consistency in the intensity of staining is particularly essential in efforts that attempt to assess the degree of tumor differentiation.

INCUBATION TEMPERATURE Because equilibrium in antigen-antibody reactions is reached more quickly at 37°C compared to room temperature, some workers prefer to incubate at the higher temperature. An increase in incubation temperature allows for a greater dilution of the antibody or a shortened incubation time. It is not known whether temperature promotes the antigen-antibody reaction selectively rather than the various reactions that give rise to background.

A temperature of 4°C is frequently used in combination with overnight or longer incubations. Slides incubated for extended periods or at 37°C should be placed in a humidity chamber to prevent evaporation and drying of the tissue sections. Similarly, tissue incubated at room temperature in a very dry or drafty environment will require the use of a humidity chamber.

REFERENCES

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2. Boenisch T. Appl Immunohistochem 2001; 9(2):176-179.
3. Sternberger LA and Sternberger NH. J Histochem Cytochem 1986;34:599-605.

*The term equilibrium here denotes saturation of antigen with antibody.