# The Use of Antibodies in Immunoassays

Immunological reagents are the backbone of every immunoassay system. Immunoassays can be utilized to quantitatively and qualitatively measure minute quantities of analytes in complex biological systems. Although the concepts behind the development of an immunoassay seem straightforward, development of assays that provide false or misleading information is common when proper selection of immunoreagents is not a critical part of assay design. Immunoassays use many types of supports and detection systems. However, at the heart of every immunoassay is an antibody system that determines the level of sensitivity and specificity. The purpose of this article is to describe how antibodies function in the immunoassay and what parameters should be considered in the selection of antibodies.

## The Anatomy of Immunoglobulins

The γ-globulins were recognized as a distinct group of serum proteins by Tiselius in 1937 (1). Immunoglobulins are glycoproteins synthesized and secreted by B-lymphocytes and plasma cells. They are separated into classes based on physical characteristics including structure, size and valency. Mammalian species produce 5 classes of immunoglobulins: IgG, IgM, IgA, IgE and IgD. The basic protein structure associated with immunoglobulins contains 2 identical light chains and 2 identical heavy chains of amino acids. These monomeric chains are covalently linked by disulfide bonds. The basic structure resembles a "Y", in which each arm contains a hypervariable region where antigen binding occurs (Fig. 1). The top portion of the "Y", containing the 2 arms, is held together by 2 disulfide bonds which, when cleaved from the bottom half of the molecule, is known as the $F(ab')_2$ fragment, because it contains both antibody binding regions. The bottom portion of the molecule contains the Fc (complement binding) region (2). This portion plays a significant role in a number of important immunological reactions *in vivo* and may be the source of nonspecific reactions with respect to *in vitro* diagnostic applications.

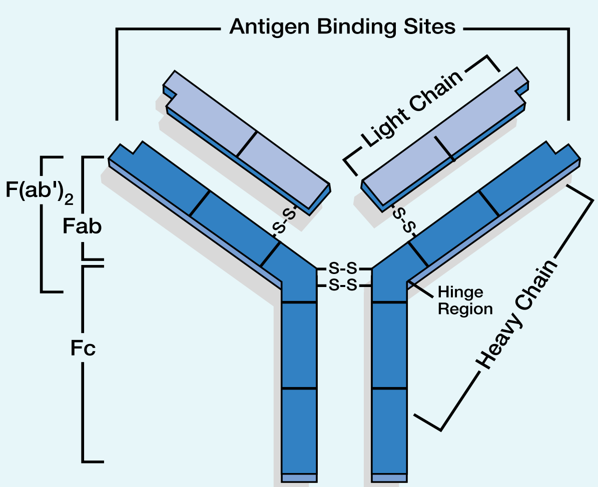


Figure 1: Structure of an IgG Antibody

Antibodies are defined functionally by the antigen they specifically recognize. Binding to antigen occurs through weak noncovalent hydrophobic and polar interactions, including Van der Waals forces. Antibody affinity is determined by the rate of formation of an antibody-antigen complex relative to the rate of its dissociation. Thus, affinity is a thermodynamic property indicative of the likelihood of antibody complex formation (3-5). In contrast, avidity is an indicator of the stability of that complex. Affinity is one determinant of avidity; others include valency of the antibody (number of antigen binding sites), valency of the antigen, and the characteristics of the antigenic determinants. Avidity is not a thermodynamic property. Rather, it can only be described functionally under given assay conditions (6-8). It is for this reason that many researchers utilize antibody titer to characterize antibody activity and concentration in a biological sample.

Antibodies are commercially available in a variety of forms, such as antiserum, ascites containing monoclonal antibody, purified immunoglobulin, and affinity purified antibody. These may vary significantly in antibody concentration, purity and heterogenicity. The antibody may be provided as a whole molecule or as an antibody fragment: $F(ab')_2$, Fc or Fab. Which antibody is chosen and in what form depends on the particular application. The goal is to choose an antibody system that provides the greatest sensitivity with the least amount of non-specific activity.

## Monoclonal Antibodies

Monoclonal antibodies are produced following the fusion of myeloma cells with antibody secreting B-cells. The resultant continuous cell line (hybridoma) produces large quantities of homogeneous, well defined, single epitope antibody (9). The availability of large quantities of continuously produced antibody allows for greater standardization and quality control of the antibody reagent. Therefore, monoclonal antibodies are more precisely characterized, legally protected and have greater acceptance by regulatory agencies when used in diagnostic applications.

Because monoclonal antibodies are the result of cell fusion, these proteins may have peculiar differences from other immunoglobulins. They may not precipitate under standard conditions. They may also demonstrate unpredictable binding patterns to Protein A and Protein G. These antibodies may not purify under ionic exchange conditions as one might expect. These properties may make the purification of monoclonal antibody from ascites a difficult and an expensive proposition. Monoclonal antibodies are also very sensitive to conjugation, and often lose activity once conjugated to enzymes such as horseradish peroxidase or alkaline phosphatase. However, one can usually conjugate monoclonal antibodies with small molecular weight molecules such as biotin with minimal risk of losing antigen-binding activity.

The advantages of using monoclonal antibodies in specific applications are numerous. Because monoclonal antibodies can be selected based on affinity during production, high affinity antibodies may be obtained. Monoclones make excellent primary antibodies in heterogeneous ELISA and other immunoassays. In competitive assays for drug, hormone or other small analytes, monoclonal antibodies are the best choice for quantitative and reproducible assays. Because of the defined specificity of the antibody reactivity these reagents can be used in epitope mapping and characterization of fine antigenic structure.

## Polyclonal Antibodies

Polyclonal antibodies are obtained from the serum of animals immunized with a particular antigen. The antibody pool obtained from serum is the result of many B-cell clones, each secreting one specific antibody. Antiserum refers to a pool of serum containing all of the antibody fraction plus other serum proteins. Due to serum proteins other than immunoglobulins, immunoassays using

## Enzyme Conjugates

Many immunodetection methods use secondary antibodies conjugated to enzymes in order to amplify the signal via the catalytic properties of the enzyme. The enzymes most commonly used for this purpose are horseradish peroxidase (HRP), alkaline phosphatase (AP), and to a lesser extent, β-galactosidase. Each enzyme offers unique features that, under the right conditions, makes it the optimal choice.

Historically, HRP substrates have been shown to be more sensitive in immunoassays compared to AP substrates. This is primarily due to the faster catalytic rate of HRP. Thus, more product is generated in a shorter incubation time (1). However, these products tend to fade after development, and they can be hazardous. Additionally, $H_2O_2$, a cosubstrate in the reaction, ultimately limits the activity of HRP by oxidizing heme iron. This can result in a shorter linear incubation period than seen for AP. In contrast, AP exhibits a slower catalytic rate, but is not self-limiting. Reaction rates remain linear over longer periods of time; therefore, sensitivity can be improved by allowing the reaction to proceed for longer incubations. AP substrates also tend to be less toxic and thus easier to handle. β-galactosidase conjugates are limited in application by a relative lack of substrate choices and becasue the size and structure of the enzyme make it difficult to conjugate to antibodies while maintaining activity and solubility.

Another factor influencing the choice of conjugate is the presence of endogenous enzyme activity in the sample which may interfere with the assay and/or increase background signal (2). Mammalian tissues often exhibit peroxidase activity, particularly in cells of hematopoietic lineage (blood cells, macrophages, etc.). In these tissues, AP conjugates present a distinct advantage. Alternatively, different isoenzymes of alkaline phophatase are expressed in many tissues. AP conjugates are typically prepared using the intestinal isoenzymes, which are not inhibited by levamisole. Thus, it is often possible to inhibit endogenous AP activity by levamisole pretreatment. The obvious exception is in intestinal tissue, where HRP would be the conjugate of choice.

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2. Bratthauer, G.L. (1994). in Javois, L.C., ed. Methods in Molecular Biology, Vol. 34: Immunocytochemical Methods and Protocols. Humana Press Inc., Totowa, NJ, 155-164.

unpurified antiserum components usually exhibit high background, poor dynamic range, and low sensitivity. Antibody purified from antiserum is obtained by selective precipitation and various forms of chromatography. An IgG fraction may only contain 10% specific antibody and is only slightly more purified than antiserum. However, this fraction is void of many serum proteins that can interfere with immunoassays.

An affinity purified antibody is one that has been purified from the IgG fraction by affinity chromatography to a selected antigen. Affinity purified antibodies exhibit the highest specificity and sensitivity that can be obtained from a circulating serum antibody pool. These antibodies exhibit specific activity to a population of antigenic determinants including continuous and discontinuous antigenic sites (8, 10). Affinity purified antibodies are useful as primary and secondary antibodies in heterogeneous immunoassays and make excellent secondary anti-species (e.g., goat anti-human IgG) antibody conjugates. Anti-bacterial membrane or viral coat proteins are directed to multiple antigenic determinants. Therefore, unlike monoclonal antibodies, affinity purified antibodies can be used as both the capture and detection antibody in capture immunoassay systems.

Although affinity purified polyclonal antibodies have many advantages, they are not the antibody of choice for some immunoassay systems. In competitive assays designed for measuring drugs or small molecular weight analytes, polyclones are not as reliable as monoclonal antibodies. Because an affinity purified polyclonal antibody contains multiple individual antibodies with varying affinities for an antigenic determinant, the affinity constant cannot be accurately determined (8). Multiple antibody affinities may increase the assay variation about a standard curve in comparison to an assay designed with single monoclonal antibody. Affinity purified antibodies require consistent antiserum quality. Due to the normal variation in the animals producing the antibody, there is greater variation in the final activity of an affinity purified polyclonal antibody than a monoclonal antibody. For systems that require exact reproducibility, monoclonal antibodies may be a better choice than polyclonal. For assays requiring broad spectrum specificity to large molecular weight antigens, affinity purified polyclonal antibodies are a clear choice.

## Antibody Fragments

Antibodies can be enzymatically digested into $F(ab')_2$, Fc, and Fab components. The Fc fraction does not contain a specific antigen-binding domain and contains regions to which serum and bacterial proteins may bind. $F(ab')_2$ antibodies have the same valency as normal IgG but have a reduced molecular weight. The Fab fragment is half the molecular weight as $F(ab')_2$ and has valency to only one epitope. The lack of an Fc portion may decrease background from unwanted serum protein binding and increase stability of the antibody-antigen complex, while the reduced size may permit more rapid diffusion of the molecule into complex samples (i.e. tissues and cells). Higher sensitivity may be obtained from $F(ab')_2$ than whole molecule antibodies due to the total decrease in mass while maintaining the same antibody valency. Because the Fab antibody has only one valency, the secondary bonds formed during the antigen-antibody complex may not be as stable as that of the $F(ab')_2$ in some applications (2-3, 5, 11). In applications where nonspecific activity due to other serum proteins is of major concern, the $F(ab')_2$ is the antibody of choice over a whole molecule.

## Summary

A wide variety of antibody products are available to the researcher designing an immunoassay. Each has advantages and disadvantages in specific applications. The goal of the researcher is to find the best antibody product for each application, minimizing non-specific reactions while increasing sensitivity and dynamic range. To accomplish this goal, one must be cognitive of the underlying physical chemistry involved in antibody-antigen interaction during each phase of the immunoassay to obtain meaningful and reliable results.

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