TECHNICAL NOTE

How KPL Purifies Its Antibodies

Antibodies are a key element in immunoassays They are the driving force behind specificity and sensitivity. Affinity purification of antiserum yields antibodies with the ultimate in specificity and sensitivity. It eliminates all other potentially interfering serum proteins and antibodies that will increase the background and cause cross-reactivity. It leaves behind a population of antibodies that only react with the epitopes chosen for inclusion on the affinity matrix. The flexibility of engineering the affinity matrix to include or eliminate certain epitopes means that the antibodies KPL provides have only the reactivities that we specify, while cross reactions are reduced to a defined and reproducible minimum.

Advantages of Affinity Purified Antibodies

- Lot-to-lot consistency. Natural variances in animal-to-animal serum pools are minimized, resulting in more standardized antibody lots. Less time is required for assay optimization with new lots.
- High specificity with the ability to select for or eliminate undesired specificities.
- Selection for high affinity antibody increases sensitivity, lowers background and reduces nonspecific reactions.
- Reduces the amount of material needed as compared to IgG fractions produced by Protein A, Protein G and other forms of IgG partitioning systems. Antibody directed to antigen is 10 times more active with affinity purification than in IgG fractions.

Antiserum

As a mixture of antibodies from many different B cell clones, an antiserum is referred to as polyclonal. Each antibody molecule within the mix has a high degree of specificity for a single epitope, but the mix has reactivity to many epitopes. Most antisera demonstrate reactivities to epitopes that are not part of the planned immunization but are due to the animal's response to pathogens it has been exposed to over its lifetime. Over the course of a planned immunization, antibodies reactive to the epitopes that are injected become dominant and others become only a minor part of the antiserum.

Another source of cross-reactivity lies in the specificity of each antibody molecule. The strength of the interaction between the antibody binding site and the epitope lies in part in the complementarity of their three dimensional structures. It is further affected by hydrophobic and ionic interactions, hydrogen bonding and van der Waals forces that occur between the binding site and epitope once they have come in close proximity to one another. It is easy to imagine two different large multi epitopic protein molecules with epitopes having similar though not identical three dimensional shapes. In fact it is also not hard to imagine these two proteins sharing an identical epitope. This cause of cross-reactivity is a characteristic of both polyclonal and monoclonal antibodies.

Immunization

KPL prepares most of its affinity purified antibodies by using highly purified normal immunoglobulins as the injected antigen in most cases. The use of normal IgG, IgM or IgA as immunogen instead of monoclonal immunoglobulins from myeloma proteins yields an antiserum that has broad reactivity to all subtypes of the normal IgG, IgM or IgA. With few exceptions KPL also immunizes animals with complete antibody molecule (IgG) instead of the Fc fragment. Although this method necessitates further purification and lowers the yield, the resultant antibody is assured of reacting broadly with the target antigen.

Selection of Antisera

KPL selects antiserum for the purification process that exhibits high activity. We test all incoming serum pools by immunodiffusion and ELISA to select for high titers of specific antibody.

Affinity Purification

Antiserum contains many lipids and serum proteins. The most prevalent among them is albumin which accounts for 50% of the protein in serum. The first step in KPL's affinity purification process is accomplished by a series of salt precipitation steps in which 99% of these substances are depleted. The resultant IgG fraction is likely to contain only 5-10% specific antibody. Once the antiserum is purified to an immunoglobulin fraction, affinity purification using both positive and negative selection is utilized to purify the specific antibody from the nonspecific antibody.

For example, if we start with an IgG fraction derived from an anti-mouse IgG antiserum, the positive selection process starts with passing the IgG fraction over an agarose column to which we have covalently attached highly purified mouse IgG. The antibodies that do not bind to the column and are washed away have either no or low affinity for mouse IgG. Once all unbound antibody is washed away, the column is treated with

a buffer that will disrupt the interaction between the antibody and the epitope. This releases the bound antibody, which is washed off the column and treated with a neutralizing buffer. The result is an affinity purified antibody referred to as anti-mouse IgG (H+L) because the pool has reactivity to both mouse Ig heavy and light chains. However, a large multi epitopic molecule such as a mouse IgG can have epitopes with similar three dimensional structures to human and/or other mammalian IgGs and to other serum proteins. If left in the pool these antibodies could contribute to background depending on the assay. These reactivities are removed by passing the antibody over an agarose column to which we have covalently attached human serum proteins and is referred to as negative selection. The resultant antibody is now referred to as anti-mouse IgG (H+L) human serum absorbed. Alternatively, negative selection can be performed with serum proteins of other mammalian species or combinations of species.

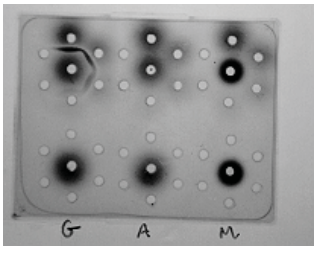
In addition to reacting with mouse IgG, the antibody preparation we have been describing will react with mouse IgM and IgA due in part to the presence of common light chains on these immunoglobulins and to common epitopes shared by gamma, mu and alpha heavy chains. This cross reactivity can be removed by passing the antibody over a column bearing mouse IgM and/or IgA. The unbound fraction is then referred to as anti-mouse IgG (γ).

Finished Product

The purified antibody is characterized by radial immunodiffusion (RID) for class cross-reaction, SDS- PAGE (Figure 1) for IgG purity, and relative potency (RP) ELISA for functionality (Figure 2). The RP assay is used to compare the final product to a reference antibody in a parallel line assay utilizing linear regression. This assay allows for characterization of both the potency and epitope recognition of the final antibody. Final stage antibody products are lyophilized and undergo testing for potency and solubility.

Antibody Purity by SDS-PAGE and RID

SDS PAGE



RID Pictures

Figure 1.

Potency Testing

Potency testing is performed on antibodies following purification and conjugation with an enzyme, fluorophore, biotin or gold. In most cases the testing is performed by ELISA and compares the new lot to both standard reference and the last lot made (Figure 2).

Cross Reactivity Testing

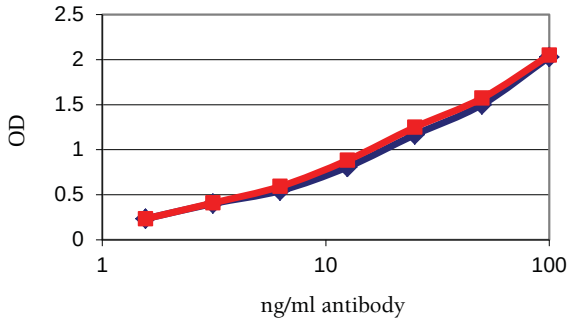


Figure 2. Comparison of a new lot of goat anti-human IgG(γ) to the reference goat anti-human IgG(γ). Varying concentrations of each antibody from 100ng/ml to 1.5ng/ml were incubated on wells of a single plate coated with human IgG, followed by a biotinylated monoclonal anti goat Ig followed by Streptavidn-HRP. Regression analysis of the curves yields sets of points wih parallel lines. Comparisons of these lines are used in quality control of each lot.

The amount of cross-reactivity is also determined in an ELISA by comparing the reactivity of an antibody with the antigen for which it is specific versus an unreactive antigen. For example, anti-mouse IgG (γ) does not react with light chains. This reactivity has been removed by adsorption with mouse IgM and IgA.

Antibody Conjugates

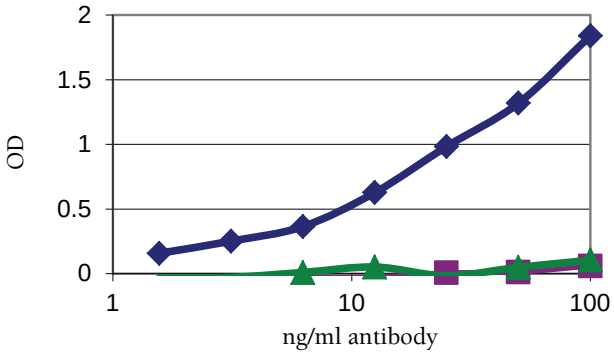


Figure 3. Comparison of reactivity of goat anti-human IgG(γ) with human IgG, IgM and IgA. Varying concentrations of goat anti-human IgG(γ) from 100ng/ml to 1.5ng/ml were incubated on wells of a single plate coated with either human IgG, IgM or IgA, followed by a biotinylated monoclonal anti goat Ig followed by Streptavidn-HRP. Regression analysis of the curves yields sets of points wih parallel lines. Comparisons of these lines are used in quality control of each lot. In the comparison of reactivity to IgG vs IgM or IgA there are no sets of points that yield a parallel line.

Identification of the desired analyte in an immunoassay via antigen-antibody interaction requires conjugation of the antibody with a label. In immunodetection assays conjugated antibodies are used both to generate signal and to amplify the system. Common labels for antibodies include enzymes, fluorophores, biotin and gold.

Enzymes

Antibodies are conjugated to enzymes to amplify the signal via the catalytic properties of the enzyme. The enzymes most commonly used for this purpose are alkaline phosphatase (AP) and horseradish peroxidase (HRP). AP and HRP conjugates can be used in microwell ELISA, membrane blotting and immunohistochemistry applications, and are detected using chromogenic and chemiluminescent substrates.

Horseradish Peroxidase

HRP and Conjugates

Horseradish peroxidase (HRP) is a holoenzyme with hemin as the prosthetic group. Removal of the iron from HRP by EDTA will inactivate the enzyme. The molecular weight is approximately 40,000 daltons with 8 carbohydrate side chains that are typically used to conjugate HRP to an antibody or streptavidin. KPL conjugates HRP to its antibodies by periodate activation of the HRP. HRP is stable for several years when lyophilized and stored at 4°C either as the native enzyme or as an antibody/streptavidin conjugate . In solution HRP and its conjugates are stable for up to a year at 1.0 mg/ml at 4°C in a suitable buffer. Dilute solutions, in the range of 0.1 mg/ml, are not stable and lose significant activity in weeks. This loss of activity at low concentrations can be minimized by stabilizing buffers. In addition, HRP is inactivated in the presence of >100mM phosphate buffers. This inactivation can be minimized by using citrate or phosphate buffers at 20mM. It has also been reported that HRP and its conjugates are inactivated by exposure to polystyrene. This can be prevented by the addition of Tween-20 to the buffer. HRP is also inactivated by exposure to azide. Avoid the use of azide in buffers.

The reaction scheme of peroxidase involves the oxidation of the enzyme by H_2O_2 to form an intermediate referred to as HRP I. HRP I is in turn reduced by a hydrogen donor via a one electron transfer to form another intermediate (HRP II) and a donor radical. HRP II is further reduced by an additional hydrogen donor via a one electron transfer to regenerate the original enzyme and another donor radical. The two donor radicals combine to yield a detectable product. In the presence of excess H_2O_2 additional intermediates (HRP III and IV) may be generated which lead to inactive peroxidase. The optimal pH for this reaction is approximately 5.0. The advantage of using HRP is its high rate constant which generates signal quickly. The disadvantage is that HRP is inactivated by excess substrate, which leads to loss of signal generation at later time points. The consequence of this is less significant when using chromogenic substrates, as once signal is generated, it continues. However, with chemiluminescent substrates one is detecting light that is emitted at that instant. HRP-based chemiluminescent substrates emit a diminished signal and results are less sensitve after 1 -2 hours than 5 - 10 minutes after adding substrate.

HRP Substrates

A variety of aromatic phenols or amines can serve as the hydrogen donor. 2,2-azino-di (3-ethyl-benzathiazoline) sulphonic acid (ABTS) and 3,5,3',5'- tetramethylbenzidine (TMB) are the two most popular chromogenic substrates. ABTS yields a blue-green soluble colored product useful in ELISA with an absorbance maximum at 405 nm. ABTS has a slower turnover rate than does TMB so its reaction rate is significantly slower. However, the dynamic range is very broad so it is an adequate substrate to use if sensitivity is not an issue. TMB yields a soluble blue colored product, useful in ELISA, with an absorbance at 650 nm. If TMB is acidified to stop the reaction, it turns yellow with an absorbance maximum at 450 nm and a 2 -3 fold increase in absorbance. Typically TMB has a detection limit 10 - 50 times lower than ABTS. While the detection limit is more a function of the antibodies being used, TMB can easily detect in the range of 0.1 - 0.3 ng/ml of HRP-labeled IgG.

Other less commonly used chromogenic substrates for HRP are o-phenylenediamine (OPD), o-dianisidine (ODIA) and 5-aminosalicylic acid (5AS).

HRP chemiluminescent substrates are generally based either on luminol or acridinium esters. HRP is first oxidized by H_2O_2 as occurs with chromogenic substrates. Luminol, a cyclic diacylhydrazide, is then oxidized by HRP to a radical which forms an endoperoxide that spontaneously decomposes to a dianion which emits light as it returns to its ground state. This process can be enhanced by phenolic compounds that intensify both the light emission and duration of the signal. The light emission has a maximum at 425 nm which can be captured by photodiode- or photomultiplier- based luminometers. This substrate can be used in both ELISA and blotting applications.

Alkaline Phosphatase

AP and Conjugates

Alkaline phosphatases are zinc metaloenzymes with 2 atoms of zinc per molecule. The molecular weight is approximately 100,000 daltons depending on the source of the enzyme. Zinc

is found in the active site and is required for activity. Removal of the zinc inactivates the enzyme. AP catalyzes the hydrolysis of an orthophosphoric monoester to yield an alcohol and an orthophosphate. Optimal catalysis occurs at approximately pH 9. Typical buffers for use with AP conjugates are tris, borate and carbonate. One should note that the high concentration of Pi in phosphate buffers acts as an inhibitor of AP and should be avoided when using AP as the enzyme. AP has a lower rate constant than HRP so turnover of substrate occurs more slowly. However, there is no substrate inhibition of AP allowing the reaction to continue for days. This is most easily seen in the case of the chemiluminescent substrates.

AP Substrates

The typical ELISA substrates for AP are *p*-nitrophenyl phosphate (*p*NPP) which yields a soluble yellow colored product with an absorption maximum at 450 nm. Another alternative is to use *p*NPP in tablet form and dissolve a tablet in buffer just before use. An alternative substrate is a formulation of bromo-chloro-indoxyl phosphate (BCIP) that has been rendered soluble. The advantage of BCIP over *p*NPP is that it is very stable, will not suffer an increased background with storage at 4° C. In addition, it is approximately 2 fold more sensitive than *p*NPP. BCIP/NBT is a precipitating formulation for use in Western blotting.

Biotin

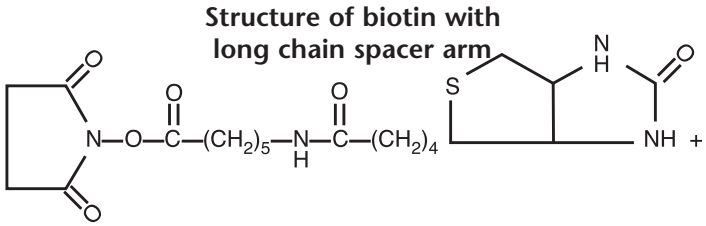


Figure 4.

Biotin, a small, water soluble vitamin, can be readily conjugated to a number of biological molecules. Antibodies labeled with biotin provide the user with a tool for increasing the sensitivity of an assay by its ability to amplify a given reaction. Biotin-labeled antibodies require the use of conjugated streptavidin or avidin. A biotin/streptavidin (avidin) system may offer significant improvement in immunodetection due to its high sensitivity and low background. The high affinity bond formed between biotin and streptavidin (avidin) ($Kd=10^{-15}$ M^{-1}) minimizes nonspecific interactions with other proteins and permits more complete washing without risk of lowered signal due to antibody detachment. The result is low background and enhanced sensitivity. KPL uses a long carbon spacer arm (Figure 4) to minimize steric hindrance between the biotinylated antibody and the streptavidin and to allow formation of a more stable biotin/streptavidin complex.

Fluorophore Conjugates

Antibodies are conjugated with fluorochromes for direct visualization o the target. The availability of fluorochromes with different emission spectra makes it possible to detect two or more antigens in the same specimen. Common fluorochromes used for labeling antibodies are fluorescein (FITC), tetramethylrhodamine (TRITC), Texas Red®, R-Phycoerythrin (R-PE) Alexa fluors and cyanine (Cy™) dyes. Fluorochrome-labeled antibodies are widely used in immunohistochemistry, flow cytometry, *in situ* hybridization, and immunofluorescent assays (IFA). Fluorescent labeling offers sensitivity and high resolution. Detection requires the use of instrumentation such as a fluorescent microscope, fluorescent plate reader or flow cytometer.

Gold Conjugates

Antibodies can also be conjugated to colloidal gold particles of different sizes. The use of gold can increase sensitivity and provide improved resolution over other methods when used on membrane or in cells and tissue. Colloidal gold may be detected with a light microscope following silver enhancement. Its use in sensitive, rapid, diagnostic immunoassays has increased greatly in the past few years. Colloidal gold conjugates are now commonly used in both electron and light microscopy, Western blots and *in situ* hybridization. KPL conjugates antibodies with colloidal gold using a modified proprietary method.

These conjugates have some advantages over enzyme conjugates including:

- Gold conjugates are smaller than enzyme conjugates and thus able to penetrate tissue better.
- Gold conjugates are not affected by endogenous peroxidases or phosphatases
- Gold conjugates are more stable then enzyme conjugates.

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