

biotinylated protein using precoated streptavidin microtiter wells. The possibility exists that the number and types of epitopes exposed after coating can be a function of the method used to coat the antigen. In some cases differences have been reported between passively adsorbed antigens and biotinylated/streptavidin coated antigens and thus it is up to each laboratory to determine which antigen coating method is best for its application. A special post coating/blocking reagent (Cat.# IM107) is provided to minimize nonspecific binding of assay reagents to the wells while stabilizing the antigen so that coated strips can be stored for long periods of time without significant loss of activity. After the plates have been coated and blocked, they are ready to use in assaying for the presence of specific antibody in samples. Samples are first diluted in the special sample diluent provided, Cat.# IM104. Typically samples are assayed at several dilutions to better semi-quantitate the amount of antibody present. Dilution is necessary to minimize nonspecific background signal while also insuring that the specific signal for most samples will be within the analytical range of the assay and microtiter plate reader. Each laboratory may need to establish optimal sample dilutions for its assay needs but in general dilutions of ~1:100 are adequate for most applications. The sample diluent has been formulated to further minimize false positive reactions. These diluted samples are then reacted in coated microtiter strips to allow for binding of any patient antibodies to the coated protein. After a wash step to remove any unbound sample components, the strips are then reacted with an antibody to human IgG h & l chains, Cat.# IM23, conjugated with the enzyme horse radish peroxidase and diluted in the conjugate diluent, Cat.# IM103 provided in the kit (suggested dilution range of 1:100 - 1:400). Because of the h & l chain reactivity of this antibody it will cross-react to varying and lesser degrees with the other classes of human immunoglobulins, IgA, IgD, IgE, and IgM. After another wash step the substrate tetramethyl benzidine (TMB), Cat.# IM105 is added and incubated for 30 minutes followed by the addition of stop solution, Cat.# IM106, to terminate the reaction. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of antibodies present. Due to difficulties in absolute quantitation in conventional ELISA formats assay data has traditionally been considered semi-quantitative with results typically reported in titer units. **Cygnus Technologies now offers a custom service to prepare and quantitate specific antibody to your product protein thus allowing you to report patient response in concentration units such as µg/mL.** If you chose to use conventional titer for the qualitative reporting of patient response, titer can be defined as the reciprocal of the sample dilution yielding a statistically significant signal above a range or “cutpoint” usually established on normal or non-immune samples. A good alternative to cutpoint analysis is to obtain a pre-therapy sample from each patient and then compare post-therapy samples to each patient’s own pre-therapy sample.

Reagents & Materials Provided

Component	Product #
Antigen Coating Buffer	IM11
Tris buffer at ~pH 8.5, 1x100mL	
Post coat/blocking Solution	IM107
Tris buffered solution with non-mammalian protein blockers & preservatives, 1x100mL	
Uncoated, qualified, microtiter plates	IM12
Plastic microtiter strip plates for passive adsorption of antigen proteins, 5 plates containing 12x8 well strips	
Sample Diluent	IM104
Tris buffered saline with a protein matrix and preservative, 1x100mL	
Goat anti-human immunoglobulin:	IM23
HRP Conjugate, Concentrate	
Affinity purified goat F(ab') ₂ antibody to human IgG h & l chains conjugated to horseradish peroxidase in a protein matrix and preservative, 1mL	
HRP Conjugate Diluent	IM103
Tris buffered saline with non-mammalian protein additives and preservatives for use in diluting IM23 conjugate concentrate, 1x100mL	
Wash Concentrate (20X solution)	IM108
Tris buffered saline with preservatives 20X concentrate for use in washing plates after coating and after each assay step, 2x50mL	
TMB Substrate	IM105
3,3',5,5' Tetramethyl benzidine, 2x50mL	
Stop Solution	IM106
0.5N sulfuric acid, 2x50mL	

Storage & Stability

- * All reagents should be stored at 2°C to 8°C until the expiration date printed on the label. **Do not freeze!**
- * When using the conjugate diluent provided with this kit, conjugates diluted to their final assay concentration will normally be stable for at least 12 months when stored at 4°C.
- * The substrate reagent should not be used if its absorbance at 450nm is greater than 0.1.
- * Reconstituted wash solution is stable until the expiration date of the kit.

Limitations of the Procedure

- Users of this kit are strongly encouraged to contact our technical services department for advice on how to overcome problems in the detection of immunogenicity.
- Detection of patient antibody to human or humanized antibody products may be problematic. In some cases the anti-human IgG:HRP conjugate used in this kit may also bind to the product antibody resulting in a very high assay background. In such cases it may be necessary to substitute another anti-human conjugate that does not cross-react with your product antibody. *Cygnus Technologies* has a number of different conjugates available and should be contacted if your product is a humanized antibody. *Cygnus Technologies* also offers a custom assay development service using a direct binding assay format (non-ELISA) that will overcome any problems when the product is a human antibody. Contact

our technical services department for advice and quotes on custom assay development.

- If you wish to report your patient antibody response in quantitative units such as $\mu\text{g/mL}$ as opposed to qualitative "titer units," *Cygnus Technologies* offers a custom service to calibrate your assay. In this case, you must send a small quantity of your product protein together with a few milliliters of positive patient sera. Using our proprietary technologies we will affinity isolate and quantitate specific antibody and return this purified reference material back to you for use in preparing quantitative standards for your assay. Contact us for a quotation on how to make your assay quantitative.

Materials & Equipment Required But Not Provided

Antigen (protein) for coating
Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm
Adjustable Pipettors – capable of accurately dispensing in the ranges of 5 to 200 μL
Repeating or multichannel pipettor – 100 μL
Microtiter plate rotator (150 - 200 rpm)
Distilled water
1 liter wash/squirt bottle for diluted wash solution

Precautions

For research use only. Not for clinical or diagnostic use in human or animals.

Stop solution contains 0.5N H_2SO_4 . Avoid contact with eyes skin, and clothing. Refer to available MSDS.

At the concentrations used in this kit none of the other reagents are believe to be harmful.

This kit should only be used by qualified technicians.

Preparation of Reagents

* Bring all reagents to room temperature.

* Dilute the 50 mL contents of wash concentrate, Cat. # IM108, to 1 liter in distilled water, and label with kit lot and expiration date. Store at 4°C.

Antigen Coating Procedure

The following procedure is one which has been shown to yield good results in a variety of antibody detection assays. Users of this kit may vary some of the coating parameters to achieve desired results. This procedure is termed "passive coating" and exploits the ability of most protein antigens to spontaneously adsorb strongly and essentially irreversibly to plastic microtiter strip wells. Adsorptive coating may in some cases alter the structure of the antigen or obscure certain epitopes to which patient antibodies might otherwise bind. In such cases other methods of coating the antigen may be preferable. One

such useful method is to first biotinylate the antigen of interest and then capture it on to plates previously coated with the specific and high avidity binding protein, streptavidin. Provided that epitopes are not eliminated by biotinylation, then in theory such coating should minimize loss of other epitopes due to structural or steric inhibition. *Cygnus* manufactures an analogous kit using streptavidin coated plates (Cat.# IM20) as well as a kit for easy biotinylation of your antigen protein (Cat.# IM30).

ANTIGEN COATING PROCEDURE

1. Dilute the antigen of interest in the Antigen Coating Buffer, # IM11. For most applications a coating concentration of 1-10 $\mu\text{g/mL}$ will be optimal. Each user may want to initially titer the coating concentration to determine the optimum coating. Some components in the antigen such as detergents may interfere with adsorptive coating. Provided that the antigen can be diluted at least 100 fold by the coating buffer, most adsorptive inhibition will be overcome. If adsorptive coating is not giving acceptable results, biotinylation of the antigen is recommended with the subsequent use of streptavidin coated plates for antigen immobilization/capture.
2. Add 160 μL of diluted antigen to each well of the microtiter plate using a multichannel pipettor. Cover plates and allow to incubate for 16 to 24 hours at ambient temperature.
3. Dump contents of plate into waste. Blot and bang plates upside down over absorbent paper to remove remaining liquid.
4. Fill all wells with at least 300 μL of 20 fold diluted Wash Solution, #IM108 using either a wash/squirt bottle or multichannel pipettor. Dump, blot, and bang wash solution from the wells as in step 3. Repeat for a total of 2 washes.
5. Add 200 μL of Post coat/Blocking Solution #IM107 to all wells and allow to block for 1 hour at room temperature.
6. Dump contents, blot and bang residual solution over absorbent paper. Plates can be used immediately or air-dried overnight and then stored in moisture proof containers with desiccant at 4°C. *(When processed and stored in this way most antigen coated plates will be stable for long periods of time up to several months. Some antigens may be more labile and each user should establish stability of their coated plates.)*

Assay Procedure

The procedure given below is intended to serve as a guideline. Users may alter various parameters to achieve desired results. Consult *Cygnus Technologies* Technical Services Department for advice on how to best modify these procedures.

ASSAY PROCEDURE

1. Dilute all samples in Sample Diluent, #IM104. For most applications a dilution of 1:100 to 1:200 will serve for initial screening of samples. Reactive samples can then be subjected to multiple dilutions in subsequent assays to better establish relative titer.
2. Add 100 μ L of diluted sample to duplicate antigen coated wells as shown on your work list.
3. Cover strips. Transfer to a microtiter plate rotator and incubate for at least 1 hour at room temperature at ~180 rpm.
4. Dump contents of plate into waste. Blot and bang plates upside down over absorbent paper to remove most of the remaining liquid.
5. Fill all wells with at least 300 μ L of diluted Wash Solution, # IM108 using either a wash/squirt bottle or multichannel pipettor. Dump, blot, and bang wash Solution from the wells as in step 4. Repeat for a total of 3 washes.
6. Dilute goat anti-human immunoglobulin: HRP conjugate concentrate, #IM23 in the Conjugate Diluent, #IM103. *Suggested dilution is 1:100 but user may want to determine optimal titer and incubation times through further experimentation.*
7. Add 100 μ L of diluted conjugate to each well, cover plate. Place on a microtiter plate rotator at ~180 rpm, and incubate for 1 hour at room temperature. Dump and wash wells as described in steps 4 & 5 above for a total of 4 washes.
8. Add 100 μ L of TMB Substrate, #IM105 to all wells and allow to incubate at room temperature for 30 minutes.
9. Add 100 μ L of Stop Solution, #IM106 to terminate the reaction.
10. Read all wells on an approved microtiter plate reader at the 450 nm. If your plate reader has the capability for dual wavelength reading with automatic subtraction of a reference wavelength, a second reference wavelength of 650 nm is recommended.

Procedural Notes

- * Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. If duplicate CV's are poor, you may need to evaluate your evaluate plate washing technique. In general we do not recommend the use of automated plate washers since many of these devices will yield significantly poorer precision than can be obtained by good manual technique.
- * High Dose Hook Effect or poor dilutional linearity may be observed in samples with very high concentrations of antibodies. If a hook effect is possible, samples should also be assayed at more than one dilution. If the absorbance of the less diluted sample is less than the more diluted samples this may be indicative of the hook effect.
- * Bring all reagents to room temperature prior to use.
- * Serum is the preferred sample. Plasma samples may give slightly different values when compared to sera and may also give higher nonspecific binding.
- * All controls and samples should be assayed in duplicate.
- * Maintain a repetitive timing sequence from well to well for all assay steps to insure that all incubation times are the same for each well.
- * Make a work list for each assay to identify the location of each standard control and sample.
- * If the substrate has a distinct color prior to the assay it may have been contaminated. If this appears to be the case add 100 μ L of substrate and 100 μ L of Stop Solution to a microtiter well and read against a water blank. If the absorbance is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

Interpretation of Results

The lack of calibrated reference standards together with the fact that patient antibodies may have different affinities and be of different classes and subclasses means that absolute quantitation of antibody response in terms of mass per volume units is difficult to achieve. *Cygnus* is pleased to offer a special service to quantitate your assay. This service uses our proprietary affinity purification methods to isolate and quantitate highly purified antibody to your product for use as assay calibrators. Contact our technical services department for a description and quotation on this service. Conventional methods of expressing antibody response are semi-quantitative and involve reporting of antibody response in relative units such as titer. Titer is the reciprocal of the sample dilution that yields a certain signal deemed statistically significant.

True negative patient samples may give variations in nonspecific assay signal over a wide range due to a number of factors. For this reason it is advised when possible to obtain pre-therapy samples on all patients to establish each patient's baseline in the assay. When this is not practical it may be necessary to test a number of normal or non-immune patient samples to establish the range for negatives. Once this range or patient baseline is established a statistical "cutpoint" for positivity can then be assigned for a given antigen.

When high levels of drug are used in therapy the possibility exists that much of the circulating patient antibodies will be bound to the drug. Such drug bound immune complexes can interfere in the ability of various assays such as ELISA and Bioassay to detect drug specific antibody. In such cases it may be necessary to wait for some time after the last administration of drug before obtaining blood samples from the patient to test for antibody activity.

For most applications, a wait of a few days after the last administration of drug will be sufficient for most of the drug and its immune complexes to be cleared.

Determination of a positive antibody response to an antigen may not be sufficient information by itself. In some cases it may be informative to further characterize the nature of that immune response or to confirm positives in another assay as discussed below:

1. Bioassays in which positive sera are tested for their ability to “neutralize” the biological function of a protein may be a useful model to predict if the antibody response could compromise drug efficacy. Unfortunately bioassays may not always reflect what is happening in each patient. Other aspects of each patient’s health could determine the significance of antibodies to a given protein. Furthermore some antibodies while not biologically neutralizing may modify drug efficacy by causing more rapid clearance of the drug or promoting allergic reactions that could confuse interpretation of the patient’s response to the drug. In some cases inherent properties of the drug may provide for detection of the drug in circulation and thus may permit the performance of clearance studies. Such clearance information might then be correlated to patient immune response.

2. Confirmatory or “Blocking” assays for initially positive samples in the immunoassay are also advised. Often the cutpoints statistically established in the immunogenicity screening assay leave the potential for false positives. In many cases false positives may be identified by subjecting them to a blocking assay. This test is typically performed just as the screening immunoassay protocol, except that a sufficient excess of antigen is added to the diluted patient sample prior to reacting it with the antigen coated plate. In cases where the added antigen fails to block most of the coated plate binding, it can be concluded that this sample was a false positive due to some nonspecific factor such as rheumatoid factor, anti-heterophile antibodies etc.

3. Immunoglobulin Typing - The basic immunoassay method described in this insert can also be used to further characterize the nature of each patient’s immune response. By substituting the broadly reactive anti-human immunoglobulin h & l chain screening antibody with other more specific anti-human antibodies it is possible to identify the exact immunoglobulin class and even the IgG subclass. This information can be useful in understanding why the drug is immunogenic and how best to solve the problem. *Cygnus* sells other HRP conjugated antibodies to human immunoglobulins that can be plugged into the screening assay you have developed. These include very specific anti-human immunoglobulin class antibodies for IgA, IgD, IgE, IgG (gamma chain specific), and IgM. These can be purchased individually or may be obtained as 5 separate vials in a convenient kit form, Cat.# IM40.

Subclass specific HRP:antibody conjugates are available for the 4 different human IgG subclasses also individually or in kit form, Cat.# IM50.

4. Epitope Mapping - Once immunogenicity has been established for a given protein drug it is usually desirable to determine the antigenic epitopes on the protein responsible for eliciting the patient immune response. Epitope mapping can be performed in a variety of ways such as coating peptide fragments on to the microtiter wells or using the peptide fragments in solution phase to inhibit binding to the intact protein analogous to the **Confirmatory or Blocking** assays discussed in #2 above.

5. Adjuvant Effects from Drug Contaminants
Immunogenicity for some proteins can be elicited or enhanced by other unrelated materials co-administered with the protein through a mechanism termed the “adjuvant effect”. Thus it may be useful to test product positive samples for the presence of antibody to specific process impurities. Common bioprocess contaminants include host cell proteins from the cell expression system as well as a number of growth media additives. *Cygnus* sells many common bioprocess contaminants (antigens) that may be coated and used to test for the presence of specific patient antibodies. See our catalogue or contact customer service for a list of these bioprocess antigens.

6. Determination of Immunogenicity to Human or Humanized Antibody Products

Detection of patient antibody to human or humanized antibody products may be problematic by ELISA. The anti-human IgG:HRP conjugate used in this kit may also bind to the product antibody resulting in a very high assay background. In such cases it may be necessary to substitute another anti-human conjugate that does not cross-react with your product antibody. *Cygnus* has a number of different conjugates available and should be contacted if your product is a humanized antibody. *Cygnus* also offers a custom assay development service using a direct binding assay format (non-ELISA) that will overcome any problems when the product is a human antibody. Contact our technical services department for advice and quotes on custom assay development.

As experts in the field of immunochemical analysis *Cygnus Technologies* is available to solve your immunogenicity testing problems. In addition to a wide range of reagents and kits, we are also at your service for custom assay development.

Ordering Information/ Customer Service

To place an order or to obtain additional product information contact *Cygnus Technologies* Customer Support:

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