Protein A Assay

Immunoenzymetric Assay for the Measurement of Natural & Structurally Conserved Recombinant Protein A in samples containing Human Immunoglobulin Catalog # F050H

Intended Use

This kit is intended for use in quantitating Protein A ligands from natural *(Staphylococcus aureus)* and structurally conserved *E.coli* recombinant expressed constructs. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals. Cygnus manufactures two other Protein A detection kits, Cat #s F050 and F400. Cat # F050 does not utilize a sample treatment step and as such may not be suitable for all product antibodies due to interference from the product antibody. Protein A ELISA kit Cat # F400 incorporates some improvements over the earlier kits and is designed to better detect the newer, unnatural constructs of Protein A such as the MabSelect SuRe™ ligand sold by GE Healthcare.

Summary and Explanation

Protein A immobilized on various chromatography media is commonly used to purify antibodies. Even when covalently attached, Protein A can leach off of the chromatography support and co-elute with the antibody. For some applications such as the pharmaceutical use of the antibody, contamination with Protein A must be minimized to avoid any adverse effects.

There are several manufacturers of Protein A and Protein A chromatography supports. In addition to natural Protein A purified from *S. aureus*, there are also various recombinant constructs of Protein A typically expressed in *E. coli*. Some of these recombinant Protein A's are essentially identical to natural Protein A. However, there are other unnatural recombinant constructs with very significant structural differences when compared to natural Protein A. GE Healthcare sells one such unnatural construct marketed as MabSelect SuRe™ . Due to the unique structure of MabSelect SuRe™ it is only about 20% reactive in our F050 and F050H kits. For this reason we offer our newest Protein A kit, Cat # F400 which incorporates a new antibody as well as other changes allowing it to detect all forms of Protein A equally.

Some product antibodies when complexed with the leached Protein A can interfere in the detection of Protein A. A sample treatment step involving acid dissociation and heat denaturation is used to overcome any interference from product immunoglobulin.

The *Cygnus Technologies'* Protein A ELISA kits are designed to detect Protein A contamination to less than one part per million. As such, these kits can be used as tools to aid in optimal purification process development and in routine quality control of in-process streams as well as final product.

Principle of the Procedure

The Protein A assay is a two-site immunoenzymetric assay. Samples containing Protein A are first diluted with a sample denaturing buffer. This reagent dissociates the Protein A from the product antibody. Samples are then heated in a dry heating block or boiling water bath to denature and precipitate the product antibodies. The heat-denatured samples are then reacted in microtiter strips coated with a polyclonal anti-Protein A capture antibody. A second biotinylated monoclonal anti-Protein A antibody is simultaneously reacted forming a sandwich complex of solid phase antibody- Protein A-biotinylated antibody. After a wash step to remove any unbound reactants, the strips are then reacted with Streptavidin labeled with Alkaline Phosphatase enzyme. This reagent will bind to any of the biotinylated antibody bound to the strip. After another wash step to remove unbound Streptavidin: alkaline phosphatase, the plates are then reacted with *p*-nitrophenyl phosphate (PNPP) substrate. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of Protein A present. Accurate quantitation is achieved by comparing the signal of unknowns to Protein A standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-Protein A, biotinylated Mouse monoclonal antibody conjugated to biotin in a protein matrix with preservative. 1x12mL	**F051H**
Polyclonal Anti-Protein A coated microtiter strips 12x8 well strips in a bag with desiccant	**F052***
Protein A Standards Recombinant Protein A in a protein matrix with preservative. Standards at 0, 0.25, 1, 4, and 16 ng/mL. 1mL/vial.	**F053**
Sample Denaturing Buffer Citrate buffer with detergent and preservative. 1x20mL	**F054**
Streptavidin:Alkaline Phosphatase In a protein matrix with preservative. 1x12mL	**F009**
PNPP Substrate p-nitrophenyl phosphate in a Diethanolamine buffer with preservative. 1x12mL	**F008**
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	**F004**

*All components can be purchased separately except # F052.

Storage & Stability

* All reagents should be stored at 2°C to 8°C for stability until the expiration date printed.

* The substrate reagent should not be used if its stopped absorbance at 405nm is greater than 0.4.

* Reconstituted wash solution is stable until the expiration date of the kit.

Materials & Equipment Required But Not Provided

Microtiter plate reader spectrophotometer with dual wavelength capability at 405 & 492nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 405nm wavelength.)*
Boiling water bath or dry heating block
Microcentrifuge tubes
Microcentrifuge
Pipettors - 50µL and 100µL
Repeating or multichannel pipettor - 100µL
Microtiter plate rotator (150 - 200 rpm)
Sample Diluent (recommended Cat # I028)
Distilled water
1 liter wash bottle for diluted wash solution

Precautions

* For Research or Manufacturing use only.

* At the concentrations used in this kit, none of the reagents are believed to be harmful.

* This kit should only be used by qualified technicians.

Preparation of Reagents

* Bring all reagents to room temperature.

* Dilute wash concentrate to 1 liter in distilled water, label with kit lot and expiration date, and store at 4°C.

Procedural Notes

1. Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. We advise against the use of automated or other manual operated vacuum aspiration devices for washing plates as these may result in lower specific absorbances, higher non-specific absorbance, and more variable precision. The manual wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision. If duplicate CVs are poor or if the absorbance of the 0 standard minus a substrate blank is greater than 0.15, evaluate plate washing procedure for proper performance.

2. When dilution of samples is required, dilution should be performed in a diluent validated to yield acceptable background and not contaminated with Protein A. The diluent should also give acceptable recovery when spiked with known quantities of Protein A. *Cygnus* sells a diluent validated for this assay, Sample Diluent Buffer, Cat# I028. This is the same material used to prepare the kit standards. As the sample is diluted in I-028, its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for recovery by using them to dilute the 16ng/mL standard, as described in the "Limitations" section below.

3. High Dose Hook Effect may be observed in samples with very high concentrations of Protein A. Samples greater than 20µg/mL may give absorbances less than the 16ng/mL standard. Hook effect is indicated when absorbance of the undiluted sample is less than the diluted samples. If a hook effect is possible, samples should also be assayed diluted.

4. If the substrate has a distinct yellow color prior to performing the assay it may have been contaminated. If this appears to be the case read 200µL of substrate against a water blank. If the absorbance is greater than 0.4 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised. The PNPP substrate is very sensitive to environmental contamination. Do not leave bottle open or at room temperature for longer than is needed. Only remove as much reagent as is needed for your assay run and do not return any unused substrate back into the substrate bottle. Additional substrate can be purchased separately as Cat # F008.

Limitations

* Before reporting Protein A contamination using this kit, each laboratory should validate that the kit and assay procedure utilized yield acceptable specificity, accuracy, and precision. A suggested protocol for this validation can be obtained by contacting our Technical Services Department or our web site.

* This kit will not accurately detect certain unnatural recombinant constructs of Protein A such as the Protein A sold by GE Healthcare as MabSelect SuRe™. If you are using this Protein A construct you should use our kit Cat# F400.

* Some human and rabbit IgGs have been reported to inhibit the ability of the kit anti-Protein A antibodies to bind to Protein A resulting in an under-recovery of true Protein A contamination. While this kit has been designed to overcome such interference your product antibodies should be evaluated for any negative inhibition before reporting results as described in the "Limitations" section. If you encounter product antibody interference contact *Cygnus Technologies* for advice on how to solve this problem.

* Samples containing immunoglobulins in excess of 1mg/mL may interfere in the accurate quantitation of Protein A by giving falsely low values. When detection sensitivity allows, we recommend dilution of your samples to 1mg/mL or less of product antibody using our Cat # I028 diluent to minimize any interference.

* Certain sample matrices and product antibodies may interfere in this assay. Although the assay is designed to minimize matrix

interference, materials such as detergents in high concentration, extremes of pH (<6.0 and >8.5), very high buffer molarity, or very high protein concentrations may give erroneous results. **For these reasons we recommend that you first establish acceptable recovery in your sample matrices by performing a spike recovery experiment.** This test can be very simply performed by diluting the 16ng/mL standard 1 part to 3 parts of your sample matrix which does not contain any Protein A. This diluted standard when assayed as an unknown should give a value of 3.2 to 4.8 ng/mL. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Assay Protocol

* Bring all reagents to room temperature.

* Set-up plate spectrophotometer to read dual wavelength at 405nm for the test wavelength and 492nm for the reference wavelength. Blank the instrument using the zero standard wells after assay completion.

* All standards, controls and samples should be assayed in duplicate. Standards, controls, and samples should all be subjected to the same sample treatment procedure.

* **Pipette the Biotinylated antibody into the wells before adding the denatured samples. This will ensure that the sample is neutralized by the biotinylated antibody solution before it is exposed to the microtiter strip coated antibody.**

* Maintain a repetitive timing sequence from well to well for all assay steps to ensure 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 yellow color prior to the assay it may have been contaminated. If this appears to be the case, read 200µL of substrate against a water blank. If the absorbance is greater than 0.4 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

* Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site.

* The protocol specifies use of an approved microtiter plate shaker or rotator for the immunological steps. These can be purchased from most laboratory supply companies. Alternatively, you can purchase an approved, pre-calibrated shaker directly from *Cygnus Technologies*. If you do not have such a device, it is possible to incubate the plate without shaking however it will be necessary to extend the immunological incubation step in the plate by about one hour in order to achieve comparable results to the shaking protocol. **Do not shake during the 30-minute substrate incubation step, as this may result in higher backgrounds and worse precision.**

Procedural Modifications

* The assay is very robust such that assay variables like incubation times, sample size, and other sequential incubation schemes can be altered to manipulate assay performance for more sensitivity, increased upper analytical range, or reduced sample matrix interference. Increasing incubation time for the PNPP substrate step will, in general, increase absorbances proportionately for all wells. For example, doubling the substrate step time from 30 minutes to 60 minutes will double all ODs. Before modifying the protocol from what is recommended, users are advised to contact Technical Service for input on the best way to achieve your desired goals.

* Samples containing Protein A greater than 16ng/mL should be diluted in an appropriate diluent. (See Procedural Note # 2). Be sure to multiply diluted sample concentrations by the dilution factor when calculating your results.

Quality Control

* Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 1 ng/mL. CVs for samples < 1 ng/mL may be greater than 10%.

* For optimal performance the absorbance of substrate when blanked against water should be < 0.4.

* It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct. **You are strongly urged to make controls in your typical sample matrix using your product antibody. Controls can be aliquoted as single use vials, stored frozen for long-term stability.**

Example Data

Well #	Contents	Abs. at 405nm	Mean Abs.	ng/mL Protein A
1A	Zero Std	0.000		
1B	Zero Std	0.002	0.001	
1C	0.25 ng/mL	0.040		
1D	0.25 ng/mL	0.042	0.041	
1E	1 ng/mL	0.140		
1F	1 ng/mL	0.148	0.144	
1G	4 ng/mL	0.526		
1H	4 ng/mL	0.531	0.528	
2A	16ng/mL	1.743		
2B	16ng/mL	1.721	1.732	
2C	sample 1	0.004		
2D	sample 1	0.006	0.006	<0.25
2E	sample 2	0.144		
2F	sample 2	0.152	0.148	1.1

Sample Treatment Procedure

Failure to completely dissociate and remove the product antibody from the sample during the sample treatment step can result in under-recovery of Protein A. The usual cause of poor Protein A recovery is due small amounts of residual sample antibody remaining in the supernatant after the centrifugation step. Product antibody can re-associate with the Protein A during the assay protocol and cause under recovery. Careful adherence to the procedure below should insure full recovery of Protein A.
The heating step will typically result in a denatured protein precipitate containing the product antibody. The Protein A will be in the supernatant. Samples containing a high product antibody concentration (>4mg/mL) may yield a very large precipitate pellet making it difficult to recover sufficient supernatant for the assay. In such cases it is best to dilute the sample prior to denaturation in a neutral pH buffer (Cat #I028). Be sure to correct the assay result for any dilution factor.

1. Process all samples including the standards and controls by adding 1 part of sample denaturing buffer (Cat. # F054) to 4 parts of sample into a microfuge vial. (For example: Pipette 50µL of F054 into a microfuge tube containing 200µL of sample. These volumes will provide for at least triplicate analysis of your samples). **Mix thoroughly by vortexing.**

2. Make a small pin or needle hole in the cap of each microfuge tube to allow for venting of heated, expanded air inside the tube.

3. Place the tubes in the preheated block or flotation device and place this device into a validated 100°C dry heating block or boiling water bath for 5 to 10 minutes. While 5 minutes is adequate for most samples we have seen some samples where a 10-minute heat step improves recovery.

4. Remove the tubes, allow to cool for 5 minutes, and then centrifuge at 6000 to 15,000 x g for 5 minutes in a microcentrifuge or other adapted centrifuge. If your centrifuge is capable of rates of centrifugation higher than 6000x g a higher speed can yield a more tightly packed pellet less subject to re-suspension. Make certain your centrifuge is very well balanced. If you feel or hear any vibrations as the centrifuge accelerates or decelerates your rotor is unbalanced. A poorly balanced centrifuge will result in some of the pelleted product antibody being re-suspended. This re-suspended antibody is a frequent cause for under-recovery of Protein A. For more dilute samples a pellet may not be visible after centrifugation. Therefore, always orient the centrifuge tubes in the same way so you will know where the pellet will be. In this way you can avoid disruption of the pellet when removing the test sample. Avoid any delays in removing the supernatant for testing. Handle the tubes carefully to avoid bumping or vibrations that might re-suspend some of the pellet.

*** If you continue to have poor recovery after carefully following the procedures above it may be necessary to further dilute your sample prior to assay using our sample diluent Cat # I028. Dilution to the range of 1.0 to 0.1 mg/mL is usually sufficient to obtain acceptable recovery. Contact our very experienced Technical Service Department if you have any problems with recovery. Watch a video on how to perform sample treatment on our web site, www.cygnustechnologies.com, if you have concerns about your technique.**

Assay Protocol

1. Pipette 100µL of biotinylated anti-Protein A (#F051H) into each well.

2. Pipette 50µL of supernatant from the denatured standards, controls and samples into wells indicated on work list.

3. Cover & incubate on rotator at ~ 180rpm for 1 hour at room temperature, 24°C ± 4°.

4. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding well from a squirt bottle or by pipetting in ~350 µL. Dump and bang again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding Streptavidin: Alkaline Phosphatase.

5. Pipette 100µL of Streptavidin: Alkaline Phosphatase (#F009) into each well.

6. Cover & incubate on rotator at ~ 180rpm for 1 hour at room temperature, 24°C ± 4°.

7. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding well from a squirt bottle or by pipetting in ~350 µL. Dump and bang again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding PNPP substrate.

8. Pipette 100µL of PNPP substrate (#F008).

9. Cover & incubate at room temperature for 30 minutes. DO NOT SHAKE. (If OD's for the 16ng/mL standard (E) are <1.2, we recommend incubating for an additional 30 minutes for a total substrate incubation time of 60 minutes.)

10. Read absorbance at 405/492nm blanking on the Zero standard.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL. (See 'Limitations' section above). This data reduction may be performed through computer methods using curve fitting routines such as point-to-point, spline, or 4 parameter logistic fit. **Do not use linear regression analysis to interpolate values for samples as this may lead to significant inaccuracies!** Data may also be manually reduced by plotting the absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line. Absorbances of samples are then interpolated from this standard curve.

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. A more detailed copy of this "Validation Summary" report can be obtained by request. This validation is generic in nature and is intended to supplement but not replace certain user and product specific qualification and validation that should be performed by each laboratory. At a minimum each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your samples types containing Protein A within or above the analytical range of this assay should be evaluated for dilutional linearity to insure that the assay is accurate and does not suffer from "Hook Effect". Each laboratory and technician should also demonstrate competency in the assay by performing a precision study similar to that described below. A more detailed discussion of recommended user validation protocols can be obtained by contacting our Technical Services Department or on-line at our web site.

Precision

The data below shows both intra (n=20 replicates) and inter-assay (n=5 assays) coefficients of variation (%CVs). Each laboratory is encouraged to establish precision with its protocol using a similar study.

Intra-assay			Inter-assay		
# of tests	**Mean ng/mL**	**%CV**	**# of assays**	**Mean ng/mL**	**%CV**
20	1.1	7.0	5	1.0	8.7
20	4.1	6.1	5	4.1	6.3

Sensitivity

The lower limit of detection (**LOD**) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. The LOD is 100 pg/mL. The lower limit of quantitation (**LOQ**) is ~200pg/mL.

Spike Recovery

Various buffer matrices have been evaluated by spiking known amounts of Protein A. Because this assay is designed to minimize matrix interference most of these buffers yielded acceptable recovery (defined as between 80-120%). In general extremes in pH (<5.0 and >8.5) or salt concentration as well as certain detergents can cause under-recovery. Samples in the acid buffer used to dissociate your product antibody from your Protein A column may require neutralization to pH 7.0 to 7.5 before assay to obtain accurate results. In some cases very high concentrations of the product antibody may also cause a negative interference in this assay. Each user should validate that their sample matrices and product itself yield accurate recovery in the protocol of their choice. This experiment can be performed by spiking the 16ng/mL standard provided with this kit, into the sample in question. For example, we suggest adding 1 part of the 16ng/mL standard to 3 parts of the test sample. This yields an added spike of 4ng/mL. Any endogenous Protein A from the sample itself determined prior to spiking and corrected for by the 25% dilution of that sample should be subtracted from the value determined for the spiked sample. The added spike and recovery should be within allowable limits e.g. 80% to 120%. Should you have any problems achieving adequate spike and recovery data you are strongly urged to contact our Technical Services Department for recommendations on how to overcome sample matrix interference.

Specificity

This kit was shown to detect a natural *Staphylococcal* and structurally conserved recombinant Protein A material equally. On a molar basis, those forms of Protein A reacted essentially equally with recovery between 90 to 100%. Non-conserved, structurally unique recombinant forms of Protein A such as GE Healthcare's MabSelect SuRe™ may react much less. It is advisable to test your source of Protein A for recovery to ensure accurate quantitation by this kit.

Some human and rabbit IgGs have been reported to inhibit the ability of the kit anti-Protein A antibodies to bind to Protein A resulting in an under-recovery of true Protein A contamination. While this kit has been designed to overcome such interference your product antibodies should be evaluated for any negative inhibition before reporting results as described in the "Limitations" section. If you encounter product antibody interference contact *Cygnus Technologies* for advice on how to solve this problem.

Samples containing immunoglobulins in excess of 1mg/mL may interfere in the accurate quantitation of Protein A by giving falsely low values. When detection sensitivity allows, we recommend dilution of your samples to 1mg/mL or less of product antibody using our Cat # I028 diluent to minimize any interference.

Hook Capacity

Very high concentrations of Protein A were evaluated for the hook effect. At concentrations exceeding 20,000 ng/mL, the apparent concentration of Protein A may read less than the 16ng/mL standard. Samples yielding signals above the 16ng/mL standard or suspected of having concentrations in excess of 20,000 ng/mL should be assayed diluted.

Ordering Information/ Customer Service

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

www.cygnustechnologies.com
Cygnus Technologies, Inc.
4701 Southport Supply Rd. SE, Suite 7
Southport, NC 28461 USA
Tel: 910-454-9442
Fax: 910-454-9443
Email: techsupport@cygnustechnologies.com