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 three other Protein A detection kits, Cat #s F050, F400, and F400Z. 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 kits Cat # F400 and F400Z incorporate some improvements over the earlier kits and are 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, impurities 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 two other Protein A kits, Cat # F400 and F400Z which incorporate 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 impurities 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.

The F050H kit has been successfully qualified for most antibody based drug substances. We have seen a few problematic samples that give low recoveries in this kit as well as in other commercial kits. For this reason, you must qualify the assay for each new type of sample to demonstrate acceptable recovery. Most low recovery samples are either incomplete antibody fragments or so-called fusion proteins where that antibody structure is linked to another non-antibody molecule. Conventional sample treatment procedures are ineffective in dissociation, denaturation, and precipitation/removal of the drug substance prior to the Protein A detection assay. Any un-denatured drug substance remaining in the sample after the treatment step can re-associate with the leached Protein A resulting in under-recovery by the ELISA. To deal with these problematic samples we have developed another kit, our Cat # F400Z. This kit has been qualified using several problematic samples and found to yield acceptable recovery on all of them. The F400Z kit uses the same antibodies, standards, and sample treatment protocol as our F400 kit. The only difference is that the F400Z kit contains a 'Recovery Enhancer' solution added to the HRP conjugated anti-Protein A antibody. The 'Recovery Enhancer' works by preventing the re-association of any drug substance that is not completely removed or denatured during the sample treatment step.

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	**F052B***
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. 1x12mL	**F054R**
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 # F052B and F054R.

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 (400 - 600 rpm)
- Sample Diluent (recommended Cat # I028)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- The substrate reagent should not be used if its absorbance at 405nm is greater than 0.4.
- Reconstituted wash solution is stable until the expiration date of the kit.
- After prolonged storage, you may notice a salt precipitate and/or yellowing of the wash concentrate. These changes will not impact assay performance. To dissolve the precipitate, mix the wash concentrate thoroughly and dilute as directed in the 'Preparation of Reagents' section.

Precautions

- For Research or Manufacturing use only.
- 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 is greater than 0.300, evaluate plate washing procedure for proper performance.

2. When dilution of samples is required, dilution should be performed in a diluent qualified 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 qualified 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.

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 less than 1 ng/mL may be greater than 10%.
- For optimal performance, the absorbance of substrate when blanked against water should be less than 0.4.
- It is recommended that each laboratory assay appropriate quality control samples in each run to ensure 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.**

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.
- 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. In addition, a video demonstration of proper plate washing technique is also available in the 'Technical Help' section of our web site.
- The protocol specifies use of an approved orbital microtiter plate shaker for the immunological steps. These can be purchased from most laboratory supply companies. 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.**

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 orbital shaker at 400-600 rpm for 1 hour at room temperature, 24°C ± 4°C.

4. Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate or use of vacuum aspiration devices in an attempt to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle or by pipetting in ~350µL. Dump and tap 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 400-600 rpm for 1 hour at room temperature, 24°C ±4°C.

7. Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate or use of vacuum aspiration devices in an attempt to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle or by pipetting in ~350µL. Dump and tap 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.

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.

Limitations

Before reporting Protein A impurities using this kit, each laboratory should qualify that the kit and assay procedure utilized yield acceptable specificity, accuracy, and precision. A suggested protocol for this qualification 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 Cat # F400 or F400Z kits.
- 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 impurities. 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 (less than 6.0 and greater than 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.

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 ensure 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 (greater than 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.

Sample Treatment Procedure (cont.)

1. Process all samples including the standards and controls by adding 1 part of sample denaturing buffer (Cat. # F054R) to 4 parts of sample into a microfuge vial. (For example: Pipette 50µL of F054R 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 qualified 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. If you have concerns about your technique, a video demonstration on 'How to Denature Samples for the Protein A Kit' is available in the 'Technical Help' section of our web site.**

Example Data

Well #	Contents	Abs. at 405-490nm	Mean Abs.
A1	Zero Std	0.038	0.038
B1	Zero Std	0.038	
C1	0.25ng/mL	0.067	0.068
D1	0.25ng/mL	0.069	
E1	1ng/mL	0.167	0.164
F1	1ng/mL	0.160	
G1	4ng/mL	0.528	0.524
H1	4ng/mL	0.519	
A2	16ng/mL	2.170	2.191
B2	16ng/mL	2.211	

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. A more detailed copy of this "Qualification Summary" report can be obtained by request. This qualification is generic in nature and is intended to supplement but not replace certain user and product specific qualification and qualification 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 ensure 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 qualification 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		
# of tests	**Mean ng/mL**	**%CV**
20	1.1	7.0
20	4.1	6.1
Inter-assay		
# of assays	**Mean ng/mL**	**%CV**
5	1.0	8.7
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 (less than 5.0 and greater than 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 qualify 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.

This kit will provide accurate results on most recombinant therapeutic antibody products. You may encounter some samples that give consistent low recovery that cannot be adequately improved by simple dilution of the sample or by modifying the sample treatment procedure. In these cases, we recommend evaluating our F400Z Protein A ELISA kit. This kit has been qualified using several problematic samples and found to yield acceptable recovery on all of them. The F400Z kit uses the same antibodies, standards, and sample treatment protocol as our F400 kit. The only difference is that the F400Z kit contains a 'Recovery Enhancer' solution added to the HRP conjugated anti-Protein A antibody. The 'Recovery Enhancer' works by preventing the re-association of any drug substance that is not completely removed or denatured during the sample treatment.

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 impurities. 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

Cygnus Technologies also offers kits for the extraction and detection of CHO Host Cell DNA. The following kits are available:

- Residual Host Cell DNA extraction:

  Cat # D100W, DNA Extraction Kit in 96 deep well plate
  Cat # D100T, DNA Extraction Kit in microfuge tubes

- Extraction and PCR amplification of CHO Host Cell DNA for use with user supplied master mix:

  Cat # D555W, DNA Extraction Kit in 96 deep well plate
  Cat # D555T, DNA Extraction Kit in microfuge tubes

- Residual CHO Host Cell DNA extraction and detection using PicoGreen® dye:

  Cat # D550W, DNA Extraction Kit in 96 deep well plate
  Cat # D550T, DNA Extraction Kit in microfuge tubes

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

www.cygnustechnologies.com
Cygnus Technologies, LLC
4332 Southport Supply Rd. SE
Southport, NC 28461 USA
Tel: 910-454-9442
Fax: 910-454-9443

Email for all Order inquiries:
orders@cygnustechnologies.com

Email for Technical Support:
techsupport@cygnustechnologies.com