



Protein A Assay

Immunoenzymetric Assay for the Measurement of Natural & Structurally Conserved Recombinant Protein A Catalog # F050

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 F050H and F400. Cat # F050H is preferred over F050 when the product antibody is human or otherwise shows interference in the detection of Protein A. Our newest Protein A kit Cat # F400 incorporates some improvements over the two 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 Of the Test

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™. Because of 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. That kit 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 when using the F050 kit. Like our F050H kit, Cat # F400 also utilizes a sample treatment step involving acid dissociation and heat denaturation 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 a tool 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 reacted in microtiter strips coated with a polyclonal anti-Protein A capture antibody. A second biotinylated Monoclonal anti-Protein A antibody is 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 which 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

<u>Component</u>	<u>Product #</u>
Mouse anti-Protein A, biotinylated	F051
Mouse monoclonal antibody conjugated to biotin in a protein matrix with preservative, 1x12mL.	
Polyclonal Anti-Protein A coated microtiter strips	F052
12x8 well strips in a bag with desiccant	
Protein A Standards	F053
Recombinant Protein A in a protein matrix with preservative. Standards at 0, 0.25, 1, 4, & 16 ng/mL. 1mL/vial.	
Streptavidin: Alkaline Phosphatase	F009
In a protein matrix with preservative, 1x12mL	
PNPP Substrate	F008
<i>p</i> -nitrophenyl phosphate in a Diethanolamine buffer with preservative, 1x12mL.	
Wash Concentrate (20X)	F004
Tris buffered saline with preservative, 1x50mL.	

Storage & Stability

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

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.)
Pipettors - 50µL and 100µL
Repeating or multichannel pipettor - 100µL
Microtiter plate rotator (150 - 200 rpm)
Sample Diluent (Recommend Cygnus 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 personnel.

Preparation of Reagents

- * Bring all reagents to room temperature.
- * Dilute wash concentrate to 1 liter in distilled water, and 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.
- * This kit may not accurately quantitate Protein A in the presence of IgG from species other than mouse due to some interference factors with the antibodies to Protein A used in this kit. To insure accurate quantitation of Protein A in samples containing IgG from species such as human or rabbit it will be necessary to establish acceptable recovery in your matrix by performing the dilution study recommended in the paragraph below. If you fail to achieve acceptable recovery it may be necessary to try other *Cygnus* Protein A kits designed for problematic antibodies. Kit Cat#'s F050H and F400 incorporate an acid dissociating agent and a heat denaturation step to eliminate interferences from the product antibody in the accurate detection of Protein A.
- * 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. It is recommended to test the sample matrix for interference 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.
- * 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 yellow color prior to the assay it may have been contaminated. If this appears to be the case read 200 μ L 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.

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.

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, absorbance of the 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. These controls can be aliquoted into single use vials and stored frozen for long-term stability.**

Assay Protocol

- 1. Pipette 50 μ L of standards, controls and samples into wells indicated on work list.**
- 2. Pipette 100 μ L of the biotinylated anti-Protein A (#F051) into all wells.**
- 3. Transfer to rotator and incubate at room temperature, 24°C \pm 4°, for 1 hour at 180 rpm.**
- 4. Aspirate, wash with at least 300 μ L of diluted wash solution and aspirate. Wash a total of 4 times.**
- 5. Pipette 100 μ L of Streptavidin: Alkaline Phosphatase (#F009) into each well.**
- 6. Transfer to rotator and incubate at room temperature for 1 hour at 180 rpm.**
- 7. Dump the contents into waste or gently aspirate using a multi-channel pipetor. Blot and vigorously bang out residual liquid over low lint absorbance paper. Wash generously with diluted wash solution by flooding the wells with solution from a squirt bottle or by pipetting in ~350 μ L. Repeat for a total of 4 times. Wipe off any liquid from the bottom outside of the wells as any residue can interfere in the reading step.**
- 8. Pipette 100 μ L of PNPP substrate.**
- 9. Incubate for 30 minutes.** (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.

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	0.25ng/	0.035		
1D	0.25ng/	0.031	0.033	
1E	1ng/mL	0.124		
1F	1ng/mL	0.123	0.124	
1G	4ng/mL	0.487		
1H	4ng/mL	0.495	0.491	
2A	16ng/mL	2.150		
2B	16ng/mL	2.064	2.107	
2C	sample 1	0.005		
2D	sample 1	0.010	0.008	<0.1ng
2E	sample 2	0.127		
2F	sample 2	0.123	0.125	1ng

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 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=10 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.

# of tests	Intra-assay		# of assays	Inter-assay	
	Mean ng/mL	%CV		Mean ng/mL	%CV
20	1.0	6.3	5	1.1	8.3
20	4.2	5.4	5	4.2	6.5

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 120 pg/mL. The lower limit of quantitation (LOQ) is ~200pg/mL.

Spike & Recovery and Interference Studies

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.

Most mouse and humanized antibodies or human antibodies produced by cell culture in CHO or hybridoma cell lines will yield accurate recovery of Protein A with this kit. However natural human and rabbit IgGs have been shown 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. Your product antibodies should be evaluated for this negative interference 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* Customer Support:

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