



Protein A Assay

Immunoenzymetric Assay for the Measurement of Protein A Catalog # F400

Intended Use

This kit is intended for use in quantitating Protein A. The kit is for Research and Manufacturing use only and is not intended for diagnostic use in humans or animals.

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 applications such as the therapeutic use of the antibody, contamination with Protein A must be minimized to avoid any adverse patient effects. This immunoassay method provides sensitivity to detect Protein A contamination to less than 100pg/mL. Leached Protein A is typically bound to the product immunoglobulin present in the sample through its Fc region. This binding of Protein A to the product antibody can interfere in the accurate quantitation of Protein A in some immunoassays by inhibiting the ability of the anti-Protein A antibodies used in the assay to bind to the complexed Protein A. This inhibition can result in a significant underestimation of Protein A contamination. Such interference is highly variable from one product antibody to the next but is particularly common with many human IgGs.

There are several manufacturers of Protein A and Protein A chromatography supports. In addition to natural Protein A purified from *Staphylococcus aureus*, there are also various recombinant constructs of Protein A typically produced in *E.coli*. Some of these recombinant protein A's are essentially identical to natural Protein A. However, there are other unnatural recombinant constructs of Protein A that have very significant structural differences when compared to natural Protein A. GE Healthcare sells one such unique construct of Protein A marketed as MabSelect SuRe™. Because of the very different structure of this protein the possibility exists that some antibodies for Protein A will recognize the various constructs of Protein A differently resulting in quantitation inaccuracies. Such is the case with the two older Protein A kits manufactured by Cygnus Technologies, Cat #s F050 & F050H. These older kits recognize natural and as well as conserved recombinant Protein A with essentially the same structure as the purely natural Protein A. However, the F050 & F050H kits will underestimate the true concentration of the MabSelect SuRe™ ligand. For this reason, Cygnus has developed the F400 kit. This kit will cross react essentially 1:1 with

MabSelect SuRe™ relative to natural and highly conserved recombinant Protein A. If you are using natural or structurally conserved, recombinant forms of Protein A and have successfully validated the F050 or F050H kits you may continue to use those products as they will be manufactured indefinitely. If you are using a recombinant form of Protein A with very significant structural differences from natural Protein A such as MabSelect SuRe™ you should use the Cat # F400 kit. If you are evaluating our Protein A assays for the first time, we recommend use of Cat # F400 kit as it offers other robustness and procedural advantages over the older kits, in addition to its ability to more accurately quantitate unnatural forms of Protein A.

This kit, Cat # F400, is designed to detect all currently marketed constructs of Protein A. This kit will eliminate most product antibody inhibition and provide accurate quantitation through the use of a carefully validated sample treatment step. (See "Limitations" section). This assay is designed to provide a simple to use, precise, and highly sensitive method to detect Protein A contamination to less than 100pg/mL in the presence of up to mg/mL quantities of humanized monoclonal antibodies. As such this kit 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.

Reagents & Materials Provided

Component	Product #
Chicken anti-Protein A:HRP Chicken antibody conjugated to horseradish peroxidase in a protein matrix with preservative, 1x12mL	F401
Polyclonal Chicken 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. 7 Standards at 0, 0.1, 0.25, 0.6, 1.5, 4, & 10 ng/mL, 1mL/vial	F403
Sample Denaturing Buffer Citrate buffer with detergent and preservative, 1x20mL	F054
TMB Substrate 3,3',5,5' Tetramethyl benzidine, 1x12mL	F005
Stop Solution 0.5N sulfuric acid, 1x12mL	F006
Wash Concentrate (20X) Tris buffered saline with preservative, 1x50mL	F004

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. After a centrifugation step to pellet the denatured product antibody, the samples are then reacted in microtiter strips coated with a polyclonal anti-Protein A capture antibody. A second anti-Protein A antibody labeled directly with Horse Radish Peroxidase (HRP) enzyme is simultaneously reacted forming a sandwich complex of solid phase antibody-Protein A-HRP labeled antibody. After a wash step to remove any unbound reactants, the strips are then reacted with tetramethyl benzidine (TMB) substrate. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be 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.

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 absorbance at 450nm is greater than 0.1.
- * 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 450 & 650nm.
(If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)
Boiling water bath or dry heating block
Microcentrifuge tubes
MicroCentrifuge
Pipettors - 50µL, 100 and 200µL
Repeating or multichannel pipettor - 100µL
Microtiter plate shaker (150 - 200 rpm)
Sample Diluent (Recommend Cat # I028)
Distilled water
1 liter wash bottle for diluted wash solution

Precautions

For research and manufacturing use only. At the concentrations and volumes used in this kit, none of the reagents are believed to be harmful. This kit should only be used by qualified laboratory technicians.

Preparation of Reagents

- * Bring all reagents to room temperature.
- * Dilute the 50mL of 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. Cat #I028, Sample Diluent has been validated for use with this assay. This is the same diluent used to make the kit standards. As your sample is diluted in I028 its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for non-specific binding and acceptable spike & recovery as discussed below. Sample dilution should be performed prior to the sample denaturation step for best results.
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 10ng/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 be assayed over at least two dilutions.

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 at our web site. In general the most critical validation experiments involve spike & recovery and dilutional linearity/parallelism.
- * The ability of anti-Protein A antibodies to immunologically bind to Protein A can be affected by the type of product immunoglobulin present in the sample. In particular, certain natural, serum derived human

immunoglobulins when complexed to the Protein A can significantly reduce the binding of both the capture and HRP antibodies used in this kit. This interference is believed to be due to steric hindrances when the product antibody has been pre-bound to Protein A. Most mouse monoclonals, humanized monoclonals, and many human antibodies expressed in Chinese Hamster Ovary (CHO) cells or mouse hybridoma cell lines do not significantly inhibit and can be assayed at concentrations of 1mg/mL or greater. When detection sensitivity limits allow, simple dilution of the inhibitory product antibody in our recommended diluent Cat #I028 will usually overcome the interference. Most antibodies we have tested show no significant interference when diluted in Cat# I028 diluent in the range of 0.1 to 1mg/mL

* Samples in concentrated strong acids can interfere in the assay by lowering the assay pH to below the optimal range of 7.0 to 7.5. The HRP labeled antibody is in a strong buffer designed to neutralize most samples back to the ideal assay pH range. If there is some doubt about the pH interference of your sample you may conduct a simple test prior to performing the assay by adding 1 part of the denatured sample to 2 parts of the HRP conjugate and testing for pH using paper pH indicator strips.

* 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 1 part of the 10ng/mL standard supplied with the kit into 3 parts of your sample matrix which does not contain any or very low levels of Protein A. This diluted standard when assayed as an unknown should give a recovery value after correcting for any endogenous Protein A of ~2 to 3 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 450nm for the test wavelength and 650nm for the reference wavelength. (A 630nm filter can be substituted for the 650nm if your instrument is so equipped.)
- * All standards, controls and samples should be treated in exactly the same way. Assay all samples at least in duplicate.
- * **Pipette the HRP labeled antibody (#F401) into the wells before adding the denatured samples. This will ensure that the sample is neutralized by the HRP antibody buffer solution before it is exposed to the microtiter strip coated antibody. Pipetting of the sample into the well before the HRP**

labeled antibody, can damage the antibody coating and result in lower and more variable absorbances.

- * 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. Accomplish all steps as rapidly as possible to avoid “end of run” sequential process time differences that could cause systematic inaccuracies (See **Procedural Modifications** below.)
- * Make a work list for each assay to identify the location of each standard, control, and sample.
- * If the substrate has a distinct blue color prior to the assay it may have been contaminated. If this appears to be the case, read 100µL of substrate + 100µL of stop solution 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.
- * Thorough washing is essential to proper performance of this assay.

Procedural Modifications

*The assay is very robust such that assay variables like incubation times, and sample size can be altered to manipulate assay performance for more sensitivity, increased upper analytical range, or reduced sample matrix interference. 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 10ng/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 the results.

*Because of the multiple steps involved in sample treatment and in the assay itself, it is possible to introduce systematic effects as function of pipetting and incubation timing differences. These effects will manifest as what can be called “end of run” or “front to back” effects. In such cases what is typically seen are that samples assayed at the front of the plate give higher values than those at the end of the plate. Provided all the steps are performed quickly, end of run effects are insignificant for runs involving no more than 6 strips. When an entire plate of 12 strips is performed as a single assay, samples run at the end of the plate can show significant differences in apparent Protein A concentration due to timing differences. If you are performing runs of more than 6 strips we suggest that controls be run at the beginning of the run as well as the end, to document if end of run effects are present. End of run effects can usually be overcome in multiple strip runs by modifying the assay protocol for Steps 1 & 2 in the Assay Protocol section below. Rather than adding the HRP conjugate to the coated wells followed by addition of the denatured samples, we suggest pipetting 50µL of denatured samples into an uncoated “template” microtiter plate (not provided with the kit) in the same positions as the samples will be put into the coated plate provided with the kit. After addition of all

samples to the uncoated, template plate, pipette 100 μ L of the HRP conjugated antibody into all wells using a multichannel pipettor. Mix the contents on a microtiter plate shaker. Using a multichannel pipettor, transfer 100 μ L of the mixture from each uncoated template well into the corresponding well of the anti-Protein A coated plate, Cat #F052. Proceed with the assay as described in the Assay Protocol from Step 3 on.

Sample Treatment Procedure

1. Process all samples including the standards and controls by adding 1 part of sample denaturing buffer (Cat. # F054) to 2 parts of sample into a microfuge vial.

(For example: Pipette 100 μ L of F054 into a microfuge tube containing 200 μ L 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 minutes.

4. Remove the tubes, allow to cool for 5 minutes, and then centrifuge at ~6000 x g for 5 minutes in a microcentrifuge or other adapted centrifuge. Samples are now ready to assay.*

* 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.**

Assay Protocol

1. Pipette 100 μ L of HRP conjugated anti-Protein A (#F401) into all wells.

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

3. Cover or place into zip-lock plastic bag. Transfer to microtiter plate shaker, and incubate at 180rpm for 2 hours at room temperature, 24°C \pm 4°.

4. Dump the contents into waste or gently aspirate using a multi-channel pipettor. 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.

5. Pipette 100 μ L of substrate.

6. Incubate for 30 minutes at room temperature. Do not shake.

7. Pipette 100 μ L of Stop Solution.

8. Read absorbance at 450/650nm.

Quality Control

-Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 0.5 ng/mL. CVs for samples < 0.5 ng/mL may be greater than 10%.

- For optimal performance the absorbance of the substrate when blanked against water should be < 0.1.

- 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.

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.
1A	Zero Std	0.053	
1B	Zero Std	0.061	0.057
1C	.1 ng/mL	0.084	
1D	.1 ng/mL	0.081	0.083
1E	.25ng/mL	0.142	
1F	.25ng/mL	0.139	0.141
1G	.6 ng/mL	0.239	
1H	.6 ng/mL	0.249	0.244
2A	1.5ng/mL	0.486	
2B	1.5ng/mL	0.473	0.480
2C	4.0ng/mL	1.285	
2D	4.0ng/mL	1.327	1.306
2E	10ng/mL	2.696	
2F	10ng/mL	2.642	2.669

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL. (See Limitations Section). 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 or by accessing our Web site at www.cygnustechnologies.com. 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 show both intra (n=12 replicates) and inter-assay (n=10 assays) coefficients of variation (%CVs) for 3 control samples in the low, middle, and upper range of the standards. 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
12	0.258	8.3	10	0.253	10.0
12	1.546	2.0	10	1.496	4.6
12	4.373	5.1	10	4.046	6.1

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 ~50 pg/mL. The lower limit of quantitation (LOQ) is defined as the first dosed standard at 100pg/mL.

Spike & Recovery and Interference Studies

Each user should validate that their sample matrices and product itself yield accurate recovery. This experiment can be performed by spiking the 10ng/mL standard provided with this kit, into the sample in question. For example, we suggest adding 1 part of the 10 ng/mL standard to 3 parts of the test sample. This yields an added spike of 2.5 ng/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%.

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. In some cases high concentrations of the product antibody may also cause a negative interference. Serum derived polyclonal human and rabbit IgGs have been reported to partially inhibit the ability of some kit anti-Protein A antibodies to bind to Protein A resulting in potential under-recovery of true Protein A levels. 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* Technical Services Department for advice on how to solve this problem. When detection sensitivity limits allow, simple dilution of the inhibitory product antibody in our recommended diluent Cat #1028 will usually overcome the interference. Most antibodies we have

tested show no significant interference in the range of 0.1 to 1 mg/mL.

Specificity

This kit will detect natural *Staphylococcal* and various commercially available recombinant Protein A constructs. On a molar basis, 4 different constructs of Protein A commercially available at the time of the validation of this assay (August 2007) reacted essentially equally with recovery between 90 to 100%. However, you may want to test your source of Protein A for recovery to ensure accurate quantitation by this kit. If recovery is unacceptable contact our Technical Services Department for guidance. Substitution of standards made with your source of Protein A in place of the kit standards may be a solution.

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 10ng/mL standard. Samples yielding signals above the 10ng/mL standard or suspected of having concentrations in excess of 20,000 ng/mL should be assayed diluted.

Ordering Information

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

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