CYGNUS TECHNOLOGIES
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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

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 ™. 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 impurities 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

Component	Product #
Anti-Protein A, biotinylated Mouse monoclonal antibody conjugated to biotin in a protein matrix with preservative. 1x12mL	**F051**
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 16ng/mL. 1mL/vial	**F053**
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.

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.

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

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 impurities 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 impurities. 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 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 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 ensure 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 (less than 6.0 and greater than 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 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 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 50µL of standards, controls and samples into wells indicated on work list.

2. Pipette 100µL of biotinylated anti-Protein A (#F051) into each well.

3. Cover & incubate on rotator 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 plat 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 orbital shaker at 400 – 600 rpm for 1 hour at room temperature, 24°C±4°C.

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. Incubate at room temperature for 30 minutes. DO NOT SHAKE. (If OD's for the 16ng/mL standard (E) are less than 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, cubic 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.

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

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.

Example Data

Well #	Contents	Abs. at 405-490nm	Mean Abs.
A1	Zero Std	0.000	0.001
B1	Zero Std	0.002	
C1	0.25ng/mL	0.035	0.033
D1	0.25ng/mL	0.031	
E1	1ng/mL	0.124	0.124
F1	1ng/mL	0.123	
G1	4ng/mL	0.487	0.491
H1	4ng/mL	0.495	
A2	16ng/mL	2.150	2.107
B2	16ng/mL	2.064	

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			Inter-assay		
# of tests	Mean ng/mL	%CV	# of assays	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.

Recovery/ 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 (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.

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 impurities. 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

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

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

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