Protein A Mix-N-Go™ ELISA for Amsphere™ A+ Ligand

Immunoenzymetric Assay for the Measurement of
Amsphere™ A+ Protein A Constructs
Catalog # F1075

Intended Use

This kit is intended for use in quantitating the recombinant protein A ligands used in JSR Life Sciences' Amsphere™ A+ protein A chromatography resin series. It is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

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 applications such as the therapeutic use of the antibody, contamination with Protein A must be minimized to avoid any adverse patient effects. 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.

The Mix-N-Go Assay for JSR Life Sciences' Amsphere™ A+ Protein A kit is designed to eliminate most product antibody inhibition and provide accurate quantitation through the use of a 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 100 pg/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.

Principle of the Procedure

The Mix-N-Go assay for Amsphere™ A+ Protein A is a two-site immunoenzymetric assay. Samples containing Amsphere™ A+ Protein A ligands are first diluted in the Mix–N-Go Sample Diluent provided with the kit. The Mix-N-Go Denaturing Buffer is then added and mixed to dissociate the Protein A from the 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 Tetramethylbenzidine (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 in the sample. Accurate quantitation is achieved by comparing the signal of unknowns to Amsphere™ A+ Protein A Standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-Protein A: HRP Chicken antibody conjugated to horseradish peroxidase in a protein matrix with preservative. 1 x 12 mL	F1076
Polyclonal Anti-Protein A coated microtiter strips 12 x 8 well strips in a bag with desiccant.	F052
Amsphere™ A+ Protein A Standards Amsphere™ A+ Protein A ligand in a protein matrix with preservative. 8 Standards at 0.0, 0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10 ng/mL. 1 mL/vial	F1078
Mix-N-Go Denaturing Buffer Citrate buffer with detergent and preservative. 1 x 12 mL	F604
Mix-N-Go Sample Diluent Tris buffered saline with a protein matrix and preservative. 1 x 25 mL	I600
Stop Solution 0.5N sulfuric acid. 1 x 12 mL	F006
TMB Substrate 3, 3', 5, 5' Tetramethylbenzidine. 1 x 12 mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative. 1 x 50 mL	F004
Sample Treatment Plate Skirted 96 well PCR plate with adhesive foil seal.	F402

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on 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. 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 & 650 nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 450 nm wavelength.)*
- Pipettors – 50 µL and 100 µL
- Multichannel pipettor – 25 µL, 50 µL, and 100 µL
- Microtiter plate rotator (400 - 600 rpm)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H_2SO_4. Avoid contact with eyes, skin, and clothing.
- This kit should only be used by qualified technicians.

Preparation of Reagents

- Bring all reagents to room temperature.
- Dilute 20x 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 antibody coated plate to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. The manual wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Protein A. Samples greater than 20 µg/mL may give absorbances less than the 10 ng/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 the Protein A contamination results, each laboratory should qualify the kit for 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.
- Most mouse monoclonals, humanized monoclonals, and many human antibodies expressed in Chinese Hamster Ovary (CHO) cells or mouse hybridoma cell lines do not significantly cause inhibition.
- 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 4 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 10 ng/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.
- Very high IgG concentrations can inhibit the assay. We recommend initially diluting each sample to a protein concentration of 1 mg/mL and Protein A level within the analytical range of the curve as a starting point.

Assay Protocol

- Do not use reverse pipetting for this assay.
- Bring all reagents to room temperature.
- Set-up plate spectrophotometer to read dual wavelength at 450 nm for the test wavelength and 650 nm for the reference wavelength.
- All standards, controls and samples should be treated in exactly the same way. Assay all samples at least 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. 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.
- Thorough washing is essential to proper performance of this assay. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site.
- The protocol specifies use of an approved microtiter plate shaker or rotator for the immunological steps. These can be purchased from most laboratory supply companies. 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.**
- For best results, add Mix-N-Go Denaturing Buffer in the same direction as the replicates on the plate. For example, if the replicates are in A1 and A2 position the multichannel pipette horizontally when adding the Mix-N-Go Denaturing Buffer.
- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

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 10 ng/mL should **only** be diluted in the provided Mix-N-Go Sample Diluent (Cat. # I600). Be sure to multiply diluted sample concentrations by the dilution factor when calculating the results.

Sample Treatment

1. Prepare initial sample dilutions as required prior to sample treatment. Transfer to the appropriate wells of the STP, Cat. # F402.

2. All subsequent dilutions to be assayed can be made in the STP using Sample Diluent, Cat. # I600. Ensure final volume in every well prior to the addition of denaturing buffer is 100 µL.

3. Add 100 µL of the kit standards and controls to the wells.

4. Add 50 µL of Mix-N-Go Denaturing Buffer, Cat. # F604 to each well. Mix by pipetting up and down ~15 times. Use fresh tips for each addition.

5. Incubate on the bench for 5 – 10 minutes.

If you continue to have poor recovery after carefully following the procedure above it may be necessary to further dilute your sample prior to assay using Mix-N-Go Sample Diluent Cat. # I600. Dilution to the range of 1.0 to 0.1 mg/mL is usually sufficient to obtain acceptable recovery. Contact our experienced Technical Service Department if you have any problems with recovery.

Assay Protocol

Assay Protocol

1. Pipette 100 µL of the HRP-conjugated detection antibody, Cat. # F1076 into each well of the antibody coated microtiter plate, Cat. # F052.

2. Pipette 25 µL from the denatured standards, controls and samples into wells indicated on work list.

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 plate 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 ~350 µL. 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 substrate.

5. Pipette 100 µL of TMB substrate (Cat. # F005).

6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.

7. Pipette 100 µL of Stop Solution (Cat. # F006).

8. Read absorbance at 450 / 650 nm.

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.

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.
H1	0 ng/mL	0.040	0.037
H2	0 ng/mL	0.034	
G1	0.16 ng/mL	0.092	0.092
G2	0.16 ng/mL	0.093	
F1	0.31 ng/mL	0.142	0.145
F2	0.31 ng/mL	0.147	
E1	0.63 ng/mL	0.253	0.249
E2	0.63 ng/mL	0.245	
D1	1.25 ng/mL	0.434	0.444
D2	1.25 ng/mL	0.455	
C1	2.5 ng/mL	0.831	0.821
C2	2.5 ng/mL	0.811	
B1	5 ng/mL	1.479	1.475
B2	5 ng/mL	1.470	
A1	10 ng/mL	2.573	2.595
A2	10 ng/mL	2.617	

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 0.31-10ng/mL. CVs for samples < 0.31 ng/mL may be greater than 10%.
- 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.

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. This qualification is generic in nature and is intended to supplement but not replace certain user and product specific qualification and validation that should be performed by each laboratory. At a minimum, each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your sample 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 validation protocols can be obtained by contacting our Technical Services Department or on-line at our web site.

Precision

Both intra (n=20 replicates) and inter-assay (n=10 assays) precision were determined on 4 controls with low (~0.20ng/mL), low-medium (~0.50ng/mL), medium (~2.0ng/mL), and high (~7.5ng/mL) concentrations. The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	4.4%	6.9%
Low-medium	3.4%	6.3%
Medium	3.0%	3.4%
High	4.0%	5.6%

Sensitivity

The lower limit of detection (**LOD**) is defined as that concentration corresponding to a signal three standard deviations above the mean of the zero standard. LOD is ~0.03 ng/mL.
The lower limit of quantitation (**LLOQ**) is defined as the lowest concentration the assay can accurately measure. This is determined by testing the assay at several low concentration points and then interpolating that concentration which corresponds to a nominal recovery of +/-15% and precision of 15 -20% CV. The LLOQ is ~0.16 ng/mL.

Recovery/Interference Studies

Recovery was evaluated by adding known amounts of Protein A used to make the standards in this kit to the samples. All of these samples yielded acceptable recovery defined as between 80-120%. Each user should qualify that their sample matrices yield accurate recovery. Such an experiment can be performed by diluting the 10ng/mL standard provided with this kit into the sample matrix in question as described in the "Limitations" section.

Specificity/Cross-Reactivity

Cross reactivity has not been extensively investigated with this kit. You should evaluate components in your samples for positive interferences such as cross reactivity and non-specific binding.

Ordering Information/ Customer Service

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