Mix-N-Go Protein A Assays

Immunoenzymetric Assays for the Measurement of Protein A

Catalog # F600

and

Catalog # F610

Intended Use

These kits are intended for use in quantitating natural recombinant Protein A (F600) and unnatural Protein A constructs such as MabSelect SuRe™ (F610). These kits are for **Research and Manufacturing Use Only** and are not intended for diagnostic use in humans or animals. The F600 and F610 kits incorporate a well qualified sample treatment method to dissociate Protein A from IgG Drug Products **without the need for boiling samples and the associated centrifugation step.** These improvements increase the robustness of the method to accommodate previously problematic immunoglobulins. In doing so, the procedure has been simplified with over 1 hour eliminated from assay processing time.

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, impurities with Protein A must be minimized to avoid any adverse patient effects. Our immunoassay methods provide sensitivity to detect Protein A impurities 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 impurities. Such interference is highly variable from one product antibody to the next.

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. To address these differences, 2 separate kits have been developed so that labs using any form of Protein A can take advantage of the robust sample treatment and streamlined procedure of the Mix-N-Go Protein A ELISA format. The F600 kit is calibrated for natural and recombinant Protein A. This format is appropriate for most constructs of Protein A. The F610 kit uses natural recombinant Protein A calibrated against MabSelect Sure™. If you are using a recombinant form of Protein A with very significant structural differences from natural Protein A, you should use the Cat # F610 kit. If you are evaluating our Protein A assays for the very first time, we recommend use of the Cat # F600 or # F610 kits as these offer procedural advantages, improved robustness, and the ability to more accurately quantitate unnatural forms of Protein A when compared to Cygnus' older generation kits, (Cat # F050, F050H, F400 and F400Z). It is the responsibility of the end user to select the most appropriate kit and to qualify the kit with their sample types for acceptable accuracy and specificity.

The Mix-N-Go Protein A kits are designed to detect all currently marketed constructs of Protein A. They will eliminate most product antibody inhibition and provide accurate quantitation through the use of a carefully qualified sample treatment step (See 'Limitations' section). These assays are designed to provide a simple to use, precise, and highly sensitive method to detect Protein A impurities to less than 100pg/mL in the presence of up to mg/mL quantities of humanized monoclonal antibodies. 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.

Principle of the Procedure

The Mix-N-Go Protein A assays are a two-site immunoenzymetric assay. Samples containing Protein A are first diluted in the Mix–N-Go Sample Diluent provided with the kits. 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 Protein A standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-Protein A:HRP Chicken antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	**F601**
Polyclonal 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. 8 Standards at 0, 0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10 ng/mL. 1mL/vial	**F603 or F613**
Mix-N-Go Denaturing Buffer Citrate buffer with detergent and preservative. 1x12mL	**F604**
Mix-N-Go Sample Diluent Tris buffered saline with a protein matrix and preservative. 1x25mL	**I600**
Stop Solution 0.5M sulfuric acid. 1x12mL	**F006**
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12mL	**F005**
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	**F004**
Sample Treatment Plate Skirted 96 well PCR plate with adhesive foil seal	**F402**

*All components can be purchased separately except # F052 and F604.

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.

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- 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 450 & 650nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)*
- Pipettors - 50µL and 100µL
- Multichannel pipettor – 25µL, 50µL, and 100µL
- 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.

Procedural Notes

1. Complete washing of the antibody coated plate 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.

2. 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 the Protein A impurities results, 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 at our web site. In general, the most critical qualification 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 and can be assayed at product concentrations of up to 5 mg/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 4 parts of the HRP conjugate and testing 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.
- Very high IgG concentrations can inhibit the assay. This method has demonstrated excellent recovery in IgG concentrations up to 5 mg/mL. We recommend initially diluting each sample to a protein concentration of 1 mg/mL and Protein A in the analytical range of the curve as a starting point.

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.

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.
- 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.
- 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. 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 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.**

- 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.

Assay Protocol

1. Pipette 100µL of the anti-Protein A:HRP detection antibody (#F601) into each well of the antibody coated microtiter plate (#F052).

2. Pipette 25µL of 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 substrate.

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

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

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

8. Read absorbance at 450/650nm.

Example Data

F600 Standard Curve			
Well #	**Contents**	**Abs. at 450-650nm**	**Mean Abs.**
A1	Zero Std	0.029	0.028
A2	Zero Std	0.027	
B1	0.16 ng/mL	0.080	0.078
B2	0.16 ng/mL	0.077	
C1	0.31ng/mL	0.138	0.136
C2	0.31ng/mL	0.135	
D1	0.63 ng/mL	0.257	0.260
D2	0.63 ng/mL	0.264	
E1	1.25ng/mL	0.488	0.489
E2	1.25ng/mL	0.491	
F1	2.5ng/mL	0.880	0.899
F2	2.5ng/mL	0.917	
G1	5ng/mL	1.800	1.790
G2	5ng/mL	1.780	
H1	10ng/mL	3.017	3.050
H2	10ng/mL	3.083	
F610 Standard Curve			
Well #	**Contents**	**Abs. at 450-650nm**	**Mean Abs.**
A1	Zero Std	0.024	0.024
A2	Zero Std	0.024	
B1	0.16 ng/mL	0.060	0.060
B2	0.16 ng/mL	0.059	
C1	0.31ng/mL	0.095	0.094
C2	0.31ng/mL	0.094	
D1	0.63 ng/mL	0.145	0.155
D2	0.63 ng/mL	0.164	
E1	1.25ng/mL	0.311	0.311
E2	1.25ng/mL	0.312	
F1	2.5ng/mL	0.603	0.616
F2	2.5ng/mL	0.630	
G1	5ng/mL	1.182	1.177
G2	5ng/mL	1.172	
H1	10ng/mL	2.461	2.431
H2	10ng/mL	2.400	

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 **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

Sample Treatment Procedure

1. Prepare initial sample dilutions as required prior to sample treatment. Transfer to the appropriate wells of the Sample Treatment Plate (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 is 100µL.

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

4. Add 50µL 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 procedures 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 very experienced Technical Service Department, if you have any problems with recovery.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 0.16 – 10 ng/mL. CVs for samples < 0.5 ng/mL may be greater than 10%.
- 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.

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 or by accessing our web site at www.cygnustechnologies.com. 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 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 qualification 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=16 replicates) and inter-assay (n=2 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.

F600 Kit		
Intra-assay		
# of tests	Target (ng/mL)	%CV
16	7.5	5.8
16	3	6.4
16	0.5	3.6
Inter-assay		
# of assays	Target (ng/mL)	%CV
2	7.5	6.3
2	3	8.8
2	0.5	6.7
F610 Kit		
Intra-assay		
# of tests	Target (ng/mL)	%CV
16	7.5	5.6
16	3	5.6
16	0.5	7.1
Inter-assay		
# of assays	Target (ng/mL)	%CV
2	7.5	6.1
2	3	7.4
2	0.5	8.2

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. The LOD is ~50 pg/mL. The lower limit of quantitation (**LLOQ**) is defined as the lowest concentration at which recovery is within 20% of the nominal level and the repeatability is within 20% of the CV. The LLOQ of these methods is 100pg/mL.

Spike & Recovery/Interference Studies

Each user should qualify 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 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 these assays are 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. While the kits have been designed to overcome such interferences, 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 Mix-N-Go Sample Diluent Cat # I600 will usually overcome the interference.

Specificity/Cross-Reactivity

The F600 kit will detect natural *Staphylococcal* and various commercially available recombinant Protein A constructs. The F610 kit will detect unnatural Protein A constructs such as MabSelect SuRe™. However, you may want to test your source of Protein A for recovery to ensure accurate quantitation by these kits. 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.

Gentaur

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