CYGNUS TECHNOLOGIES
part of Maraval LifeSciences

J.T.Baker® BAKERBOND® PROchievA™ Protein A Mix-N-Go™

Immunoenzymetric Assay for the Measurement of
Avantor J.T.Baker® BAKERBOND® PROchievA™
Catalog # F965

Intended Use

This kit is intended for use in quantitating the recombinant Protein A ligands used in the Avantor J.T.Baker® BAKERBOND® PROchievA™ chromatographic resins. It is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

PROchievA™ is affinity chromatographic resin prepared by immobilizing recombinant Protein A ligand on agarose particles for purification of antibodies. Even when covalently attached, very small amount of Protein A ligand can leach off the chromatography support and co-elute with the antibody. For applications, such as the therapeutic use of the antibody, Protein A impurity level must be minimized and quantified. 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 an underestimation of Protein A impurities. Such interference may be variable from one product antibody to the next.

The Mix-N-Go Protein A kit is designed to eliminate most product antibody inhibition and provide accurate quantitation through the use of a carefully qualified sample treatment step (See 'Limitations' section). This assay is designed to provide a simple to use, precise, and highly sensitive method to detect Avantor J.T.Baker® BAKERBOND® PROchievA™ Protein A impurity to less than 800 pg/mL in the presence of up to 5 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 #
Anti-PROchievA™:HRP Chicken antibody conjugated to horseradish peroxidase in a protein matrix with preservative. 1x12mL	F966
Polyclonal Anti-PROchievA™ coated microtiter strips 12x8 well strips in a bag with desiccant.	F967*
Avantor J.T.Baker® BAKERBOND® PROchievA™ Protein A Standards Avantor J.T.Baker® BAKERBOND® PROchievA™ Protein A ligand in a protein matrix with preservative. 7 Standards at 0, 0.8, 2, 5, 10, 20, and 40ng/mL. 1 mL/vial	F968
Mix-N-Go™ Denaturing Buffer Citrate buffer with detergent and preservative. 1x12 mL	F604
Mix-N-Go Sample Diluent A Tris buffered saline with a protein matrix and preservative. 1x25 mL	I800
Stop Solution 0.5M sulfuric acid. 1x12mL	F006
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12 mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50 mL	F004
Sample Treatment Plate Skirted 96 well PCR plate with adhesive foil seal.	F402

*All components can be purchased separately except Cat. # F967

Principle of the Procedure

The Mix-N-Go™ assay for Avantor J.T.Baker® BAKERBOND® PROchievA™ Protein A is a two-site immunoenzymetric assay. Samples containing Avantor J.T.Baker® BAKERBOND® PROchievA™ 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 unknown samples and controls to Avantor J.T.Baker® BAKERBOND® PROchievA™ 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 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.

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 wash concentrate to 1 liter in distilled water, label with kit lot and expiration date, and store at 4°C.

Procedural Notes

1. This residual Protein A assay utilizes the proprietary Mix-N-Go sample treatment procedure. The Mix-N-Go technology universally and irreversibly dissociates Protein A and human IgG. Traditional boil and centrifuge methods require large sample dilutions (>1:1,000) for the dissociation to be effective. As such, the analytical range of the curve had to be extremely sensitive to detect residual Protein A. Due to the superior dissociation of the Mix-N-Go technology over traditional methods, samples can be tested using minimal dilution and product concentrations greater than 1 mg/mL. This allows for the analytical range to be higher, leading to a very sensitive and much more robust method.

2. 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 than automated plate washers.

3. High Dose Hook Effect may be observed in samples with very high concentrations of Protein A. Samples greater than 580ng/mL may give absorbances less than the 40ng/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 impurity results, each laboratory should qualify the kit for 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 do not significantly cause inhibition and can be assayed at product concentrations of up to 5mg/mL.
- 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. **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 40ng/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 ~7.5 to 12.5ng/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 5mg/mL. We recommend initially diluting each sample to a protein concentration of 5 mg/mL and Protein A level within the analytical range of the curve as a starting point. If interference is observed, dilution of the sample to a protein concentration of 1 mg/mL can be performed.

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 A, Cat. # I800. 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 procedure above, it may be necessary to further dilute your sample prior to assay using Mix-N-Go Sample Diluent A Cat. # I800. Dilution to the range of 1.0 to 0.1mg/mL is usually sufficient to obtain acceptable recovery. Contact our experienced Technical Service Department if you have any problems with recovery.

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 (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. In addition, a video demonstration of proper plate washing technique is available in the ‘Technical Help’ section of our website.
- 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 HRP-conjugated detection antibody, Cat. # F966 into each well of the antibody coated microtiter plate, Cat. # F967

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

3. Cover & incubate on the 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 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 (Cat. # F005).

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

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

8. Read absorbance at 450 / 650nm.

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.

Procedural Modifications

- Samples containing Protein A greater than 40ng/mL should **only** be diluted in the provided Mix-N-Go Sample Diluent A (Cat. # I800). Be sure to multiply diluted sample concentrations by the dilution factor when calculating the results.

Example Data

Well #	Contents	Abs. at 450-650 nm	Mean Abs.
A5	0ng/mL	0.131	0.129
A6	0ng/mL	0.127	
B5	0.8ng/mL	0.237	0.224
B6	0.8ng/mL	0.210	
C5	2ng/mL	0.378	0.369
C6	2ng/mL	0.360	
D5	5ng/mL	0.670	0.667
D6	5ng/mL	0.663	
E5	10ng/mL	1.204	1.218
E6	10ng/mL	1.232	
F5	20ng/mL	1.957	1.942
F6	20ng/mL	1.926	
G5	40ng/mL	2.805	2.787
G6	40ng/mL	2.769	

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 2 ng/mL. CVs for samples less than 2 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 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 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 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=20 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.

Avantor J.T.Baker® BAKERBOND® PROchievA™ Protein A JWT203 Ligand		
Intra-assay		
# of tests	**Target (ng/mL)**	**%CV**
20	15	4.5
20	6	6.9
20	3	6.2
Inter-assay		
# of assays	**Mean ng/mL**	**%CV**
10	14.5	6.0
10	6.1	6.7
10	3.0	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 ~100 pg/mL for Avantor J.T.Baker® BAKERBOND® PROchievA™ Protein A ligand. 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 this method is 700pg/mL for Avantor J.T.Baker® BAKERBOND® PROchievA™ Protein A ligand.

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 40ng/mL standard provided with this kit, into the sample in question. For example, we suggest adding 1 part of the 40ng/mL standard to 3 parts of the test sample. This yields an added spike of 10ng/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 (less than 5.0 and greater than 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 kit has 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 A Cat. # I800 will usually overcome the interference.

Specificity/Cross-Reactivity

The Cat. # F965 kit was designed to detect Avantor J.T.Baker® BAKERBOND® PROchievA™ Protein A ligand. Samples containing other ligands will yield a result lower than expected due to molecular weight of the construct and binding when compared to the Avantor J.T.Baker® BAKERBOND® PROchievA™ Protein A ligand assay calibrators. Thus, any lab using any other Protein A ligand must use suitable kit.

Hook Capacity

Very high concentrations of Protein A were evaluated for the hook effect. At concentrations exceeding 580 ng/mL, the apparent concentration of Protein A may read less than the 40ng/mL standard. Samples yielding signals above the 40ng/mL standard or suspected of having concentrations in excess of 580ng/mL should be assayed diluted.

Ordering Information/ Customer Service

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

www.cygnustechnologies.com
Cygnus Technologies, LLC
1523 Olde Waterford Way
Leland, NC 28451 USA
Tel: 910-454-9442

Email for all Order inquiries:
orders@cygnustechnologies.com

Email for Technical Support:
techsupport@cygnustechnologies.com
