CYGNUS TECHNOLOGIES
part of Maravai LifeSciences

Mix-N-Go Assay for Tosoh R50, R40 and R28 Protein A

Immunoenzymetric Assay for the Measurement of
Tosoh R50, R40 and R28 Protein A Constructs
Catalog # F910

Intended Use

This kit is intended for use in quantitating the recombinant Protein A ligands used in the Tosoh Bioscience TOYOPEARL® SuperA (R50), TOYOPEARL AF-rProtein A HC-650F (R40), or TOYOPEARL AF-rProtein A-650F (R28) chromatographic resins. When using this assay for TOYOPEARL Super A (R50) or TOYOPEARL AF-rProtein A-650F (R28) resin, an adjustment is necessary for assay calibration (see Specificity/Cross Reactivity section). 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, impurities 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 impurities. Such interference is highly 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 Tosoh R50/R40/R28 Protein A impurity 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.

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	F911
Polyclonal Anti-Protein A coated microtiter strips 12x8 well strips in a bag with desiccant.	F052
Tosoh R40 Protein A Standards Tosoh R40 Protein A ligand 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. 1 mL/vial	F913
Mix-N-Go Denaturing Buffer Citrate buffer with detergent and preservative. 1x12 mL	F604
Mix-N-Go Sample Diluent Tris buffered saline with a protein matrix and preservative. 1x25 mL	I600
Stop Solution 0.5M sulfuric acid. 1x12 mL	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

Principle of the Procedure

The Mix-N-Go assay for Tosoh R50/R40/R28 Protein A is a two-site immunoenzymetric assay. Samples containing Tosoh R50, Tosoh R40, or Tosoh R28 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 Tosoh R50, Tosoh R40, or Tosoh R28 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. At the concentrations used in this kit, none of the other reagents are believed to be harmful.
- 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 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 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 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 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. 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 level within the analytical range of the curve as a starting point.

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 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

- 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. (A 630 nm filter can be substituted for the 650 nm 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 100 µL of the HRP-conjugated detection antibody, Cat. # F911 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 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 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 ~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.

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.

Example Data

Well #	Contents	Abs. at 450-650 nm	Mean Abs.
A1	0ng/mL	0.102	0.101
A2	0ng/mL	0.100	
B1	0.16ng/mL	0.184	0.186
B2	0.16ng/mL	0.188	
C1	0.31ng/mL	0.255	0.257
C2	0.31ng/mL	0.258	
D1	0.63ng/mL	0.395	0.392
D2	0.63ng/mL	0.388	
E1	1.25ng/mL	0.678	0.676
E2	1.25ng/mL	0.673	
F1	2.5ng/mL	1.261	1.244
F2	2.5ng/mL	1.227	
G1	5ng/mL	1.810	1.851
G2	5ng/mL	1.892	
H1	10ng/mL	2.843	2.897
H2	10ng/mL	2.951	

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%.
- 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=8 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.

Tosoh R40 Protein A JWT203 Ligand		
Intra-assay		
# of tests	**Target (ng/mL)**	**%CV**
8	5.0	5.8
8	1.5	3.2
8	0.3	6.3
Inter-assay		
# of assays	**Mean ng/mL**	**%CV**
2	5.0	6.1
2	1.5	3.9
2	0.3	4.7

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 ~ 30 pg/mL for Tosoh R50 Protein A ligand, ~ 50 pg/mL for Tosoh R40 Protein A ligand, and 70 pg/mL for Tosoh R28 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 60 pg/mL for Tosoh R50 Protein A ligand, 100 pg/mL for Tosoh R40 Protein A ligand, and 140 pg/mL for Tosoh R28 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 10 ng/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. 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 Cat. # I600 will usually overcome the interference.

Specificity/Cross-Reactivity

The Cat. # F910 kit will detect multiple Tosoh Protein A ligands. Samples containing the Tosoh R28 ligands will yield a result ~30% lower than expected due to molecular weight of the construct when compared to the R40 assay calibrators. Thus, any lab using the R28 ligand must multiply the sample results by 1.3 to get an accurate measurement of the Tosoh R28 ligand. Samples containing the Tosoh R50 ligands will yield a result ~50-60% higher than expected. Thus, any labs using the R50 ligand must multiply the results by ~0.6 to get an accurate measurement of the Tosoh R50 ligand.

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 10 ng/mL standard. Samples yielding signals above the 10 ng/mL standard or suspected of having concentrations in excess of 20,000 ng/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
Fax: 910-454-9443

Email for all Order inquiries:
orders@cygnustechnologies.com

Email for Technical Support:
techsupport@cygnustechnologies.com
