CYGNUS TECHNOLOGIES
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Mix-N-Go™ Assay for KANEKA KanCapA™ 3G Protein A

Immunoenzymetric Assay for the Measurement of KANEKA KanCapA™ 3G Protein A Construct
Catalog # F950

Intended Use

This kit is intended for use in quantitating the recombinant Protein A ligand using the KANEKA KanCapA™ 3G chromatographic resin. The kit 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 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 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 KANEKA KanCapA™ 3G Protein A impurity up to 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 KANEKA KanCapA™ 3G Protein A is a two-site immunoenzymetric assay. Samples containing KANEKA KanCapA™ 3G 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 KANEKA KanCapA™ 3G 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. 1x12mL	F951
Polyclonal Anti-Protein A coated microtiter strips 12x8 well strips in a bag with desiccant	F052*
KANEKA KanCapA™ 3G Protein A Standards KANEKA KanCapA™ 3G Protein A ligand in a protein matrix with preservative. Standards at 0, 0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10ng/mL. 1 mL/vial	F953
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
Sample Treatment Plate Skirted 96 well PCR plate with adhesive foil seal.	F402
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

*All components can be purchased separately except # F052and F604.

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
- Repeating or multichannel pipettor - 100µL
- Microtiter plate rotator (400-600 rpm)
- Distilled water
- 1 liter wash bottle for diluted wash solution

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.

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 plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. We advise against the use of automated or other manually 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.

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 dilution linearity.
- 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.
- Avoid the assay of samples containing sodium azide (NaN_3) which will destroy the HRP activity of the conjugate and could result in the under-estimation of KANEKA KanCapA™ 3G Protein A levels.

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 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 of proper plate washing technique is available in the 'Technical Help' section of our web site.
- The protocol specifies use of an approved orbital 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 (#F951) 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 - 600rpm 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

Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.123	0.125
A2	Zero Std	0.126	
B1	0.16ng/mL	0.196	0.192
B2	0.16ng/mL	0.188	
C1	0.31ng/mL	0.237	0.231
C2	0.31ng/mL	0.225	
D1	0.63ng/mL	0.333	0.335
D2	0.63ng/mL	0.336	
E1	1.25ng/mL	0.575	0.570
E2	1.25ng/mL	0.564	
F1	2.5ng/mL	0.953	0.977
F2	2.5ng/mL	1.000	
G1	5ng/mL	1.560	1.507
G2	5ng/mL	1.454	
H1	10ng/mL	2.431	2.471
H2	10ng/mL	2.510	

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL. 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 in the range of 0.16-10ng/mL. CVs for samples less than 0.16ng/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.

Performance Characteristics

Cygnus Technologies has qualified this assay using the most recent International Code of Harmonization analytical criteria. Any new sample types must be qualified by your lab to determine MRD and acceptable spike & recovery as described above and in our Qualification Summary report. Operators should refer to that report for specifics on methods used in qualification, and expected assay performance. This qualification is generic in nature and is intended to supplement but not replace a comprehensive user and sample type qualification that should be performed by each laboratory.

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 ~20 pg/mL.

The lower limit of quantitation (LLOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are less than 20%. The LLOQ is ~0.16ng/mL.

Specificity/Cross-Reactivity

Cross reactivity to non- KANEKA KanCapA™ 3G Protein A components 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. Negative interference studies are described below.

Precision

Both intra (n=16 replicates) and inter-assay (n=2 assays) precision were determined on 3 pools with low (~0.5ng/mL), medium (~2ng/mL), and high concentrations (~5ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Intra-assay		
# of tests	Target ng/mL	% CV
8	5.0	6.6
8	2.0	7.7
8	0.5	8.7
Inter-assay		
# of assays	Mean ng/mL	% CV
2	5.0	10.1
2	2.1	9.9
2	0.5	9.2

Recovery/Interference Studies

Controls with known concentrations of KANEKA KanCapA™ 3G Protein A were used to evaluate the performance of this assay. All of these samples yielded acceptable recovery defined as between 80-120%. The standards used in this kit contain 4mg/mL of bovine serum albumin intended to simulate non-specific protein effects of most sample proteins. However, very high concentrations of some products may interfere in the accurate measurement of KANEKA KanCapA™ 3G Protein A. In general, extremes in pH (less than 5.0 and greater than 8.5), high salt concentration, high polysaccharide concentrations, urea, organic solvents, and most detergents can cause under-recovery. 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. *Cygnus Technologies* offers a more concentrated form of the KANEKA KanCapA™ 3G Protein A used to prepare the kits standards for your spike recovery and preparation of analyte controls.

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the detection of HCPs from CHO cells, as well as extraction and detection of CHO Host Cell DNA. The following kits are available:

Cat# F550 CHO HCP ELISA, 3G

- Residual Host Cell DNA extraction:

Cat # D100W, DNA Extraction Kit in 96 deep well plate
Cat # D100T, DNA Extraction Kit in microfuge tubes

- Extraction and PCR amplification of CHO Host Cell DNA for use with user supplied master mix:

Cat# D555W, DNA Extraction Kit in 96 deep well plate
Cat # D555T, DNA Extraction Kit in microfuge tubes

- Residual CHO Host Cell DNA extraction and detection using PicoGreen® dye:

Cat # D550W, DNA Extraction Kit in 96 deep well plate
Cat # D550T, DNA Extraction Kit in microfuge tubes

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

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