CYGNUS TECHNOLOGIES
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Staphylococcus aureus Host Cell Proteins

Immunoenzymetric Assay for the Measurement of *Staphylococcus aureus* Host Cell Proteins Catalog # F320

Intended Use

This kit has been specifically developed and qualified for use in detecting *Staphylococcus aureus* HCPs in Protalex's Protein A product. 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 as naturally produced by ***S. aureus*** has found many useful biological applications. It is widely used as an affinity ligand to purify antibodies and also found utility as a diagnostic and therapeutic agent. Even highly purified Protein A may contain detectable levels of other ***S. aureus*** proteins hereafter referred to as Host Cell Proteins (HCPs). When the Protein A is to be used for *in-vivo* diagnostic or therapeutic applications in humans and animals, HCP impurities must be reduced to the lowest levels practical to avoid adverse toxic or immunological reactions.

Immunological methods using antibodies to HCPs such as Western Blot and ELISA are widely accepted due to their specificity and sensitivity. While Western blot is a powerful method aiding in the identity of HCPs, it suffers from a number of limitations. Western blot is a complex and technique dependent procedure requiring a subjective interpretation of results. Furthermore, it is essentially a qualitative method and does not lend itself to obtaining quantitative answers. The sensitivity of Western blot is severely limited by the volume of sample that can be tested and by interference from the presence of high concentrations of the intended product. Western Blot may be able to detect HCPs in samples from upstream in the purification process but it often lacks adequate sensitivity to detect HCPs in purified downstream and final product. The microtiter plate immunoenzymetric assay (ELISA) method employed in this kit overcomes the limitations of Western blots providing on the order of 100 fold better sensitivity. This simple to use, highly sensitive, objective, and semi-quantitative ELISA is a powerful method to aid in optimal purification process development, process control, and in routine quality control and product release testing. This kit is "generic" in the sense that it is intended to react with essentially all of the HCPs that could contaminate the product independent of the purification process. The antibodies have been generated against and affinity purified using conditioned media to obtain HCPs typically encountered in initial product recovery steps. Because of the high sensitivity and broad reactivity of the antibodies, this generic kit has been successfully qualified for testing of final product HCPs as well as in-process samples. The suitability of this kit for sample types not previously qualified must be established experimentally by each laboratory.

Principle of the Procedure

The *Staphylococcus aureus* Host Cell Protein assay is a two-site immunoenzymetric assay utilizing a "reverse sequential protocol". In this protocol the samples, standards, and controls are first incubated in uncoated test tubes or small microfuge vials together with HRP enzyme labeled *S. aureus* antibodies for 1 hour. After this first incubation the reactant mixture is then transferred to anti-HCP antibody coated microtiter wells and incubated for another hour. The microtiter strips are then washed to remove any unbound reactants. The substrate tetramethylbenzidine (TMB) is then reacted for 30 minutes. After the addition of a "stop" reagent, the amount of hydrolyzed substrate is read on a microtiter plate spectrophotometer. The intensity of the colored substrate product will be directly proportional to the concentration of *S. aureus* proteins present in the sample.

Reagents & Materials Provided

Component	Product #
Anti-*S. aureus*:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	**F321**
Anti-*S. aureus* coated microtiter strips 12x8 well strips in a bag with desiccant	**F322***
***S. aureus* HCP Standards** Growth media derived HCPs in bovine serum albumin with preservative. Standards at 0, 5, 20, 75, 200, and 500ng/mL. 1 mL/vial	**F323**
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**

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 & 650nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)*
- Pipettors – adjustable or fixed volume, at 100, 125, and 200µL
- Repeating or multichannel pipettor - 100µL
- Microtiter plate rotator (400 - 600 rpm)
- Sample Diluent (recommended Cat # I028)
- 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. 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 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. If duplicate CVs are poor or if the absorbance of the 0 standard is greater than 0.200, evaluate plate washing procedure for proper performance.

2. High Dose Hook Effect may be observed in samples with very high concentrations of HCP. High Dose Hook Effect is due to insufficient excess of antibody for very high concentrations of HCPs present in the sample. Samples with greater than 250 µg/mL may give absorbances less than the 500 ng/mL standard. It is also possible for samples to have certain HCPs in concentrations exceeding the amount of antibody for that particular HCP. In such cases the absorbance of the undiluted sample may be lower than the highest standard in the kit however these samples will fail to show acceptable dilutional recovery/ parallelism as evidenced by an apparent increase in HCP concentration with increasing dilution. High Dose Hook is most likely to be encountered from samples early in the purification process. If a hook effect is possible, samples should also be assayed diluted. If the HCP concentration of the undiluted sample is less than the diluted sample this may be indicative of the hook effect. Such samples should be diluted at least to the minimum required dilution (MRD) where the dilution adjusted value remains essentially constant. The HCP value to be reported for such samples is the dilution corrected value at or greater than the established MRD. The diluent used should be compatible with accurate recovery. The preferred diluent is our Cat# I028 available in 100mL, 500mL, or 1 liter bottles. This is the same material used to prepare the kit standards. As the sample is diluted in I028 its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for recovery by using them to dilute the 500ng/mL standard, as described in the "Limitations" section below.

3. If the substrate has a distinct blue color prior to performing the assay it may have been contaminated. If this appears to be the case, read 100µL of substrate plus 100µl of stop against a water blank. If the absorbance is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

Limitations

- Before relying exclusively on this assay to detect host cell proteins, each laboratory should qualify that the kit antibodies and assay procedure 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.
- The standards used in this assay are comprised of *S. aureus* HCPs obtained from growth media. Western blot analysis of the antibodies used in this kit demonstrates that they recognize the majority of distinct PAGE separated bands seen using a

sensitive protein staining method like silver stain or colloidal gold. This kit has been qualified for HCP detection in sample types used in the purification procedure at the time of qualification. If this kit is to be applied to other products or sample types, it must be qualified to establish acceptable accuracy and specificity for those sample types.

- Certain sample matrices may interfere in this assay. The product protein itself, high or low pH, high salt, detergents, or other components in the sample matrix may result in either positive or negative interference in this assay. It is advised to test all sample matrices for interference by diluting the 500ng/mL standard, 1 part to 4 parts of the matrix containing no or very low HCP impurities. This diluted standard when assayed as an unknown should give a value of 75 to 125 ng/mL. Consult ***Cygnus Technologies*** Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Quality Control

- 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 HCPs derived from your cell line. These controls can be aliquoted into single use vials and stored frozen for long-term stability.**

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 10 ng/mL and less than 500 ng/mL. CVs for samples less than 10 ng/mL may be greater than 10%.

Assay Protocol

- The assay is very robust such that assay variables like incubation times, sample size, and other sequential incubation schemes 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 our technical services for input on the best way to achieve your desired goals.

- The protocol specifies the use of an approved orbital microtiter plate shaker for the immunological step. 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 microtiter plate wells by about 30 minutes to one hour in order to achieve comparable results to the 1 hour shaking protocol. Do not shake during the 30 minute substrate incubation step as this may result in higher backgrounds and worse precision.

- 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. We suggest blanking the instrument using one of the zero standard wells after assay completion.

- All standards, controls and test samples should be assayed 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.

- 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 website.

- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Use clean polypropylene test tubes or micro-centrifuge vials with caps.

2. Pipette 100µL of standards, (0-500ng/mL), controls, and samples into labeled tubes or vials as indicated on work list.

3. Pipette 200µL of anti-*S.aureus* HCP:HRP conjugate (#F321) into each tube or vial. *These volumes of 100µL for the sample and 200µL for the conjugate are recommended, assuming the assay is performed in duplicate. If assaying in triplicate or more, the relative volumes should be adjusted appropriately.

4. Cap, vortex, and allow to incubate for 1 hour at room temperature.

5. Transfer 125µL of the reaction mixture to duplicate coated wells in the anti-*S.aureus* HCP coated microtiter strips as indicated on the work list.

6. Cover or place into a zip lock plastic bag. Transfer to rotator and incubate at 400-600 rpm for 1 hour at room temperature, 24°C±4°C.

7. 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 was 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.

8. Pipette 100µL of TMB substrate (#F005) into all wells.

9. Place cover on plate and incubate for 30 minutes at room temperature. Do not shake!

10. Pipette 100µL of stop solution (#F006).

11. Read absorbance at 450/650nm.

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.

Example Data

Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.060	0.062
B1	Zero Std	0.063	
C1	5ng/mL	0.117	0.117
D1	5ng/mL	0.116	
E1	20ng/mL	0.320	0.333
F1	20ng/mL	0.346	
G1	75ng/mL	0.968	0.937
H1	75ng/mL	0.905	
A2	200ng/mL	2.029	2.002
B2	200ng/mL	1.975	
C2	500ng/mL	3.567	3.540
D2	500ng/mL	3.512	

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the extraction of Host Cell DNA. The following kits are available:

Residual Host Cell DNA extraction:

Cat # D100W DNA Extraction Kit in 96 deep well plate

Cat # D100T DNA Extraction Kit in microfuge tubes

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

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