SF9 Insect Cell Host Cell Proteins

Immunoenzymetric Assay for the Measurement of SF9 Insect Cell Host Cell Proteins Catalog # F020

Intended Use

This kit is intended for use in determining the presence host cell protein contamination in products manufactured by recombinant expression in SF9 Insect host cells. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals. Users should validate this assay for use with their product samples.

Summary and Explanation

Recombinant expression by SF9 Insect Cells is a widely used procedure to obtain sufficient and cost effective quantities of a desired protein. Many of these recombinantly produced proteins are intended for use as therapeutic agents in humans and animals and as such must be highly purified. The manufacturing and purification process of these products leaves the potential for contamination by host cell proteins from SF9 Insect Cells. Such contamination can result in adverse toxic or immunological reactions and thus it is desirable to reduce host cell contamination to the lowest levels practical. Immunological methods using antibodies to HCPs such as Western Blot and ELISA are conventionally accepted. While Western blot is a useful 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. While Western Blot may be able to detect HCPs in samples from upstream in the purification process it often lacks adequate sensitivity and specificity 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, 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 a mild lysate washed of SF9 cells to obtain HCPs typically encountered in your initial product recovery step. Western blot was used as a preliminary method and established that the antibodies reacted to the majority of HCP bands resolved by the PAGE separation. If you have need of a more sensitive and specific method to demonstrate reactivity to individual HCPs in your samples *Cygnus Technologies* recommends a method we find superior to 2D Western blot. We term this method 2D HPLC-ELISA. 2D HPLC- ELISA can yield much better sensitivity and specificity as compared to 2D Western blot. For more information on this 2D HPLC-ELISA analysis please contact our Technical Services department.

Special procedures were utilized in the generation of these antibodies to insure that low molecular weight and less immunogenic contaminants as well as high molecular weight components would be represented. As such this kit can be used as a process development tool to monitor the optimal removal of host cell contaminants as well as in routine final product release testing. Because of the high sensitivity and broad reactivity of the antibodies, this generic kit has been successfully validated for testing of final product HCPs in many different products regardless of growth and purification process. When the kit can be satisfactorily validated for your samples, the application of a more process specific assay is probably not necessary in that such an assay would only provide information redundant to this generic assay. However, if your validation studies indicate the antibodies in this kit are not sufficiently reactive with your process specific HCPs it may be desirable to also develop a more process specific ELISA. This later generation assay may require the use of a more specific and defined antisera. Alternatively, if the polyclonal antibody used in this kit provides sufficient sensitivity and broad antigen reactivity, it may be possible to substitute the standards used in this kit for ones made from the contaminants that typically co-purify through your purification process and thus achieve better accuracy for process specific HCPs. The suitability of this kit for a given sample type and product must be determined and validated experimentally by each laboratory. The use of a process specific assay with more defined antigens and antibodies in theory may yield better sensitivity however such an assay runs the risk of being too specific in that it may fail to detect new or atypical contaminants that might result from some process irregularity or change. For this reason it is recommended that a broadly reactive "generic" host cell protein assay be used as part of the final product purity analysis even when a process specific assay is available. If you deem a more process specific assay is necessary, *Cygnus Technologies* is available to apply its proven technologies to develop such antibodies and assays on custom basis.

Principle of the Procedure

The SF9 Insect Cell Host Cell Protein assay is a two-site immunoenzymetric assay. Samples containing SF9 Insect Cell proteins are reacted in microtiter strips coated with an affinity purified capture antibody. A second biotin labeled anti-SF9
Insect Cell antibody is reacted simultaneously, forming a

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sandwich complex of solid phase antibody-SF9 Insect Cell protein-enzyme labeled antibody. The microtiter strips are then washed to remove any unbound reactants. Streptavidin conjugated to HRP is added and will bind to any biotin labeled antibody previously complexed to the microtiter well. After additional washes, the substrate tetramethyl benzidine is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of SF9 Insect Host Cell proteins present.

Reagents & Materials Provided

Component	Product #
Anti-SF9 insect cell, biotinylated Affinity purified rabbit antibody conjugated to biotin in a protein matrix with preservative. 1x12mL	F094
Anti-SF9 insect cell coated microtiter strips 12x8 well strips in a bag with desiccant	F023*
SF9 insect cell HCP Standards Solubilized SF9 insect cell HCPs in bovine albumin with preservative. Standards at 0, 2, 8, 25, 75, and 200ng/mL. 1 mL/vial	F022
Streptavidin:HRP In a protein matrix with preservative. 1x12mL	F099
Stop Solution 0.5N 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 # F023.

Storage & Stability

* All reagents should be stored at 2°C to 8°C for stability until the expiration date printed.

* The substrate reagent should not be used if its stopped absorbance at 450nm is greater than 0.1.

* 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 - 50µL and 100µL
Repeating or multichannel pipettor - 100µL
Microtiter plate rotator (150 - 200 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 0.5N 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 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 minus a substrate blank is greater than 0.2, evaluate plate washing procedure for proper performance.

2. High Dose Hook Effect or poor dilutional linearity 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 samples upstream in the purification process. Samples greater than 200 µg/mL may give absorbances less than the 200 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 linearity/parallelism as evidenced by an apparent increase in dilution corrected HCP concentration with increasing dilution. High Dose Hook and poor dilutional linearity are 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 dilutions (MRDs) as established by your validation studies using your actual final and in-process drug samples. The MRD is the first dilution at which all subsequent dilutions yield the same HCP value within the statistical limits of assay precision. 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

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caused by dilutional artifacts. Other prospective diluents must be tested for recovery by using them to dilute the 200ng/mL standard, as described in the "Limitations" section below.

Limitations

* Before relying exclusively on this assay to detect host cell proteins, each laboratory should validate that the kit antibodies and assay method utilized yield acceptable specificity, accuracy and precision. A suggested protocol for this validation can be obtained by contacting our Technical Services Department or at our web site.

* The standards used in this assay are comprised of SF9 HCPs solubilized by mechanical disruption and detergent. 1D 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. Because the vast majority of HCPs will be conserved among all strains of SF9 this kit should be adequately reactive to HCPs from your strain. Other clients have successfully validated this kit for their individual SF9 strains demonstrating acceptable specificity, accuracy, and sensitivity for process intermediate samples as well as final product. However, there can be no guarantee that this assay will detect all proteins or protein fragments from your process. If you desire a much more sensitive and specific method than western blot to detect the reactivity of the antibodies in this kit to your individual HCPs *Cygnus* is pleased to offer a service for fractionation of HCPs using 2-D HPLC methods followed by detection in ELISA.

* Certain sample matrices may interfere in this assay. The standards used in this kit contain BSA at approximately 8mg/mL in a buffered saline to simulate typical sample protein and matrices. However, the potential exists that the product protein or other components in the sample matrix may result in either positive or negative interference in this assay. High or low pH, detergents, urea, high salt concentrations, and organic solvents are some of the known interference factors. It is advised to test the sample matrix for interference by diluting the 200ng/mL standard, 1 part to 4 parts of the matrix containing no or very low SF9 HCP contaminants. This diluted standard when assayed as an unknown should give a value of 30 to 50ng/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 HCP levels.

Assay Protocol

* The suggested assay protocol takes approximately 3.5 hours to complete and will yield a sensitivity of <1ng/mL. The assay is very robust such that assay variables like incubation times, sample size, and using sequential incubation schemes can be altered to manipulate assay parameters 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.

* 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.

* The protocol specifies the use of an approved microtiter plate shaker or rotator for the immunological step. These can be purchased from most laboratory supply companies. Alternatively, you can purchase an approved, pre-calibrated shaker directly from *Cygnus Technologies*. 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 first and second incubation steps by about 30 minutes each 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.**

* 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.

* All standards, controls, and samples should be assayed at least in duplicate.

* Maintain a repetitive timing sequence from well to well for all assay steps to insure 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.

* If the substrate has a distinct blue color prior to the assay it may have been contaminated. If this appears to be the case read 100µL of substrate plus 100µL of stop solution 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.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL "total immuno-reactive HCP equivalents" (See Limitations section above). 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.

Assay Protocol

1. Pipette 50µL of standards, controls and samples into wells indicated on work list.

2. Pipette 100µL of biotinylated anti-SF9 (#F094) into each well.

3. Cover & incubate on rotator at ~ 180rpm for 2 hours at room temperature, 24°C ± 4°.

4. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding well from a squirt bottle or by pipetting in ~350 µL. Dump and bang 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 Streptavidin:HRP.

5. Pipette 100µL of Streptavidin:HRP (#F099) into each well.

6. Cover & incubate on rotator at ~ 180rpm for 1 hour at room temperature, 24°C ± 4°.

7. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding well from a squirt bottle or by pipetting in ~350 µL. Dump and bang 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 TMB substrate.

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

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

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

11. Read absorbance at 450/650nm blanking on the Zero standard.

Quality Control

* Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 5-200ng/mL. CVs for samples < 5 ng/mL may be greater than 10%.

* For optimal performance the absorbance of the substrate when blanked against water should be < 0.1.

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

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL HCP equivs.
1A	Zero Std	0.000		
1B	Zero Std	0.004	0.002	
1C	2ng/mL	0.028		
1D	2ng/mL	0.024	0.026	
1E	8ng/mL	0.106		
1F	8ng/mL	0.100	0.103	
1G	25ng/mL	0.278		
1H	25ng/mL	0.298	0.288	
2A	75ng/mL	0.899		
2B	75ng/mL	0.875	0.887	
2C	200ng/mL	1.618		
2D	200ng/mL	1.678	1.648	
2E	sample A	0.021		
2F	sample A	0.029	0.025	2ng
2G	sample B	1.825		
2H	sample B	1.923	1.874	>200ng

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. This validation is generic in nature and is intended to supplement but not replace certain user and product specific qualification and validation 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 process derived HCPs within or above the analytical range of this assay should be evaluated for dilutional linearity to insure that the assay is accurate and has sufficient antibody excess for your particular HCPs. 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 validation protocols can be obtained by contacting our Technical Services Department or on-line at our web site.

Sensitivity

The lower limit of detection (**LOD**) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. LOD is 0.8 ng/mL.

The lower limit of quantitation (**LOQ**) is defined as the lowest concentration, where concentration coefficients of variation (CVs) typically are <20%. The LOQ is 1.4 ng/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=5 assays) precision were determined on 3 pools with low (2ng/mL), medium (6ng/mL), and high concentrations (70ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

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Pool	Intra assay CV	Inter assay CV
Low	14.3%	17.1%
Medium	8.0%	7.4%
High	2.5%	3.9%

Recovery/Interference Studies

Various buffer matrices have been evaluated by adding known amounts of SF9 HCPs used to make the standards in this kit. 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 (<6.0 and >8.5) as well as certain detergents can cause under-recovery. Organic solvents and high salt concentration can also interfere. In some cases very high concentrations of the product protein may also cause a negative interference in this assay. Each user should validate that their sample matrices and product itself yield accurate recovery. Such an experiment can be performed by diluting the 200ng/mL standard provided with this kit into the sample in question. For example, we suggest adding 1 part of the 200 ng/mL standard to 4 parts of the test sample. This yields an added spike of 40ng/mL. Any endogenous SF9 HCPs from the sample itself determined prior to spiking and corrected for by the 20% dilution of that sample can 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%. Should you have any problems achieving adequate spike and recovery data you are strongly urged to contact our Technical Services Department for recommendations on how to overcome sample matrix interference.

Hook Capacity

Increasing concentrations of HCPs > 200 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration that will give an absorbance reading less than the 200 ng/mL standard was >200 µg/mL.