



HEK 293 Host Cell Proteins

Immunoenzymetric Assay for the Measurement of HEK 293 Host Cell Proteins Catalog # F150

Intended Use

This kit is intended for use in determining the presence of host cell protein contamination in products manufactured by recombinant expression in HEK 293 (Human Embryonic Kidney cell line 293) host cells. The kit is for Research and Manufacturing Use Only and is not intended for diagnostic use in humans or animals.

Summary and Explanation

Recombinant expression by the HEK 293 cell line has been used for cost effective production of a number of complex proteins. When applied with viral vectors, HEK 293 cells have also shown utility as production systems for various gene therapy agents. Many of these recombinant products 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 (HCPs) from the HEK 293 cell line. Such contamination can reduce the efficacy of the therapeutic agent and result in adverse toxic or immunological reactions and thus it is desirable to reduce HCP contamination to the lowest levels practical. 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. While Western Blot may be able to detect HCPs in samples from upstream in the purification process 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 a mild lysate washed of HEK cells to obtain HCPs typically encountered in your initial product recovery step. 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.

Principle of the Procedure

The HEK 293 HCP assay is a two-site immunoenzymetric assay. Samples which may contain HEK 293 HCPs are reacted in microtiter strips coated with an affinity purified capture antibody. A second horse radish peroxidase (HRP) enzyme labeled anti-HEK 293 antibody is reacted simultaneously resulting in the formation of a sandwich complex of solid phase antibody - HCP - enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactants. The substrate tetramethyl benzidine (TMB) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of HEK 293 HCPs present.

Reagents & Materials Provided

Component	Product #
Anti-HEK 293:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative, 1x12mL	F151
Anti- HEK 293 coated microtiter strips 12x8 well strips in a bag with desiccant	F152
HEK 293 HCP Standards Solubilized HEK 293 HCPs in a bovine serum albumin matrix with preservative. Standards at 0, 1, 4, 20, 75, & 250ng/mL, 0.5 mL/vial	F153
TMB Substrate 3,3',5,5' Tetramethyl benzidine, 1x12mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative, 1x50mL	F004
Stop Solution 0.5N sulfuric acid, 1x12mL	F006

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 - 25µL 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 H₂SO₄. 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, and 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 0.300, 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 greater than 10µg/mL may give absorbances less

than the 250ng/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 I028A 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 250ng/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 procedure 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 HEK 293 HCPs solubilized by mechanical disruption and detergent. 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 HEK 293 this kit should be adequately reactive to HCPs from your strain. Several clients have successfully validated this kit for their individual 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. In recognition of this potential limitation, we suggest reporting unknown samples in arbitrary dose units such as "ng/mL or parts per million of total immuno-reactive HCP equivalents".

* Certain sample matrices may interfere in this assay. The standards used in this kit attempt 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. It is advised to test all sample matrices for

interference by diluting the 250ng/mL standard, 1 part to 4 parts of the matrix containing no or very low *HCP* contaminants. This diluted standard when assayed as an unknown should give a value of 40 to 60 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) since it will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

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 protocols specify use of an approved microtiter plate shaker or rotator for the immunological steps. 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 routine protocols. **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.

* 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.

*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.

* If the substrate has a distinct blue color prior to the assay it may have been contaminated. If you suspect significant contamination we suggest the simple experiment of reading the absorbance of 100 μL of substrate plus 100 μL of stop against a 200 μL water blank. If the substrate is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

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

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 3 ng/mL and < 250 ng/mL. CVs for samples < 3ng/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.

Assay Protocol

1. Pipette 25 μL of standards, controls and samples into wells indicated on work list.

2. Pipette 100 μL of anti-HEK 293:HRP into each well.

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

4. Aspirate, or dump the contents to waste. Blot and vigorously bang out residual liquid over absorbent paper. Wash generously with diluted wash solution by flooding the wells with solution from a squirt bottle or by pipeting 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.

5. Pipette 100 μL of substrate.

6. Incubate for 30 minutes. (Do Not Shake)

7. Pipette 100 μL of Stop Solution.

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

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.

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL HCP equivs.
1A	Zero Std	0.000		
1B	Zero Std	0.002	0.001	
1C	1ng/mL	0.026		
1D	1ng/mL	0.030	0.028	
1E	4ng/mL	0.092		
1F	4ng/mL	0.098	0.095	
1G	20ng/mL	0.316		
1H	20ng/mL	0.302	0.309	
2A	75ng/mL	0.833		
2B	75ng/mL	0.853	0.843	
2C	250ng/ml	1.702		
2D	250ng/ml	1.612	1.657	
2E	sample A	1.755		
2F	sample A	1.715	1.735	>250ng
2G	sample B	0.095		
2H	sample B	0.099	0.097	4.1ng

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. A more detailed copy of this “Validation Summary” report can be obtained by request. This validation is generic in nature and is intended to supplement but not replace certain user and product specific 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**) defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard is <0.3 ng/mL.

The lower limit of quantitation (**LOQ**) defined as that concentration where concentration coefficients of variation (CVs) are <20% is 1ng/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=5 assays) precision were determined on 3 pools with low (~3ng/mL), medium (~40ng/mL), and high concentration (~150ng/mL). The % CV is the standard deviation divided by the mean multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	9.6	14.8
Medium	7.1	7.7
High	6.8	5.4

Recovery/ Interference Studies

Various buffer matrices were evaluated by adding known amounts of HCP preparation 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), high salt concentration, and certain detergents can cause under-recovery. Your product protein or virus at high concentrations can also interfere in the assay. Each user should validate that their sample matrices yield accurate recovery. Such an experiment can be performed, by diluting the 250ng/mL standard provided with this kit, into the sample matrix in question. Where sensitivity allows, it may be possible to overcome sample matrix interference by dilution of the sample into Cat #I028 diluent.

Specificity/Cross-Reactivity

The proteins listed in the table below were tested for cross-reactivity. Positive cross-reactivity and negative-cross reactivity were evaluated by spiking the cross reactant into both the zero standard and the 250ng/mL standard. The %cross-reactivity is calculated as the apparent increase or decrease in HEK 293 HCP activity divided by the concentration of the cross reactant. Only human IgG showed any cross-reactivity.

Cross reactant	% Cross Reactivity
Human Albumin at 5mg/mL	0
Human Albumin at 5µg/mL	0
Human Transferrin, 2mg/mL	0
Human Transferrin at 2µg/mL	0
Human Insulin at 75µg/mL	0
Human Insulin at 750ng/mL	0
Human IgG at 5 mg/mL	0.008
Human IgG at 50µg/mL	0.069
Human IgG at 5µg/mL	0.014

Hook Capacity

Increasing concentrations of HCPs > 250 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 250 ng/mL standard was ~ 10 µg/mL.

Ordering Information/**Customer Service**

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

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