

HEK 293 HCP ELISA Assay

Validation Summary Catalog # F650

Summary and Explanation

The data summarized below was generated by Cygnus Technologies to establish the performance parameters and validity of this kit to measure HEK 293 cell Host Cell Proteins (HCPs). This data is intended to supplement and not replace user generated validation data. The data is representative of what a laboratory can expect to achieve when following the kit insert recommended protocols. Significant differences in these performance parameters may be indicative of problems with reagents, laboratory equipment, or technique and should be investigated before reporting results.

Our 1D western blot analysis and ELISA qualification of 6 commercial products expressed in HEK 293 including virus, vaccine and recombinant protein products indicate that most of the HCP are conserved among all cell lines. Therefore, this assay should be useful for detecting HCPs from other HEK 293 cell lines and processes. Western blot both 1 & 2 dimensional, has traditionally been used to characterize anti-HCP antibodies. Unfortunately Western blot suffers from a number of limitations. Western blot is highly orthogonal to ELISA and to non-specific protein staining methods such as silver stain or colloidal gold. Because Western blot has poor sensitivity and specificity relative to ELISA, the lack of identity between silver stain and western blot does not necessarily mean there is not antibody to that protein or that the ELISA will not detect that protein. 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 we recommend a method termed 2D HPLC-ELISA. A detailed technical discussion of this method can be found on our web site. This method has been shown to be much more sensitive and specific than 2D Western blot in detecting antibody reactivity to individual HCPs. Labs wishing to perform Western blot analysis can purchase the neat anti-HEK 293 antiserum, Cat # HK293.

It is recommended that a user validation study include at least the experiments discussed below to validate this kit for use with their product. (1) Each user should perform a study to determine antibody reactivity to individual HCPs from their process. Western blot may provide limited data in this regard but a much superior method involves ELISA analysis of 2D HPLC fractionated HCPs to demonstrate that the antibody reacts with the majority of proteins. Cygnus is pleased to offer a service for fractionation of HCPs using 2D HPLC followed by detection in the ELISA. (2) Each user should perform intra and inter assay precision experiments to establish their procedural proficiency. (3) Laboratories should also perform dilutional linearity experiments on their actual samples. This experiment assumes that samples from the purification process will have significant levels of HCPs. Such samples are to be serially diluted with

Sample Diluent Buffer, Catalog # I028 the approved diluent for this assay, or some appropriate diluent previously shown to give acceptable recovery. When diluted, samples should give essentially the same value at each dilution when multiplied by the appropriate dilution factor. This experiment establishes the minimum required dilution (MRD) at which the sample must be assayed to obtain the condition of antibody excess for accurate quantitation. (4) Once the MRD has been determined for each sample type, labs should next perform spike and recovery experiments at those MRDs. Such a study can be performed by adding known amounts of the 200ng/mL standard provided with this kit to the final product or any intermediate samples, which are to be tested. Ideally these test sample matrices should be devoid of any HEK 293 cell proteins or have very low levels (<20ng/mL) determined prior to adding the 200ng/mL standard. Such an experiment will establish the degree of sample matrix interference in the recovery of HCPs.

Materials & Methods Used

Materials	
Goat anti-HEK 293:HRP Conjugate, Lots 119, 129, and 110	Cat #F651
Microtiter coated plate, Lots 190110 & 6129	Cat #F652
HEK 293 Standards, Lots 8129 & 2089	Cat #F653
The protocol as defined in the kit insert was used in this validation.	
Data References: Raw data for these experiments are recorded in Cygnus Notebook.	#HEK #2-06 Pages 36-65
The assay method validated herein uses materials and Standard Operating Procedures (SOPs) common to the production of kits for many other analytes routinely manufactured by Cygnus Technologies. These SOPs and kits are time tested over several years, well characterized, and validated. Cygnus conducts its R&D and manufacturing operations according to the essentials of GLP and cGMP regulations and guidelines.	

Antibody Development & Characterization

The antibodies used in this kit were generated against a mild lysate of HEK 293 cells extracted by a procedure similar to those used in propagating and harvesting virus transfected cells. One dimensional Western blot of the antibodies indicate they recognize the majority of HEK 293 HCPs. Samples from in-process as well as final drug substance from 6 different products were evaluated by various experiments. These experiments indicate a high degree of conservation of HEK 293 HCP among all cell lines and processes. As such, it is believed this kit

should be of utility for strains and other HEK expressed products.

Assay Development

The assay format is a 96 well microtiter strip sandwich ELISA method using HRP as the enzyme and TMB as the substrate. The “simultaneous” assay procedure was used to generate the validation data. Microtiter plate wells are passively coated with affinity purified goat anti-HCP antibody, blocked and stabilized with Cygnus, Catalog #1044. The assay uses 6 standards ranging in concentration from 0 to 200ng/mL. HEK 293 HCPs from a non-virus transfected cell line were obtained from mild lysate of HEK 293 for use as assay standards/calibrators.

Several assay protocols were evaluated during the development of the ELISA. Sequential incubation of sample first with either the coated capture antibody (forward sequential) or first with the enzyme conjugated antibody (reverse sequential) was compared to the simultaneous assay in which both sample and conjugated antibody are incubated together. The effects of sample volume, incubation times, and antibody conjugate concentration were also evaluated in selecting the final protocol. Analysis of these variations, indicate that the assay and its antibodies are robust and that minor protocol changes should not significantly affect the accuracy of the method. Thus, it is believed that the assay protocol could be modified to specifically manipulate certain other performance parameters such as more or less sensitivity, increased analytical range, or reduced assay time. Should any laboratory using this kit decide to modify the assay protocol it is recommended that they perform a validation study similar to that described below.

Sensitivity

Limit of Detection (LOD) - The HEK 293 cell HCP concentration corresponding to an OD signal 2 standard deviations above the mean of the zero standard is defined as the LOD. This was determined from 20 replicates of the zero standard. The mean signal of the zero standard plus 2SD yielded a LOD of 727 pg/mL as interpolated from the straight line plot of the mean ODs for the zero and 4ng/mL standards.

Limit of Quantitation (LOQ) - LOQ is defined as the lowest concentration for which the CV is typically <20%. This is determined by performing a precision profile on control samples at 2ng/mL and 4ng/mL. The %CV for 20 replicates of the 2ng/mL control was 18.5 %. The %CV for 20 replicates of the 4ng/mL control was 11.1%. This data suggests an LOQ of ~2 ng/mL.

Standard Curve

Typical standard curve data from an actual assay run using a point to point fit is shown below. Actual OD values may change from lab to lab, run to run, or lot to lot. For this reason, we do not recommend use of OD levels as absolute QC parameters. The most important QC parameter involves the use of real analyte controls assayed in each run across the relevant analytical range of the assay. Do not rely on your curve fit algorithm parameters to quality control this assay. Those parameters such

as R², slope, intercept, upper and lower asymptotes etc. are too indirect and insensitive to provide critical analytical control.

Standard	Duplicate OD Values	Mean OD	%CV
0ng/mL	0.167 0.181	0.174	5.7
4ng/mL	0.227 0.222	0.225	1.6
10ng/mL	0.292 0.302	0.297	2.4
25ng/mL	0.483 0.473	0.478	1.5
75ng/mL	1.051 1.011	1.031	2.7
200ng/mL	2.011 2.056	2.034	1.6

Precision

Precision is defined as the percent coefficient of variation (%CV). This is calculated by dividing the standard deviation by the mean for a number of replicate determinations of three different control samples in the low, mid and high concentration range of the assay. Both within (intra-assay) and between (inter-assay) precision were determined. The design goal specifications are given in the last column of each experiment. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results. For labs having difficulty in routinely achieving these specifications it is suggested they assay all samples at least in triplicate to better identify statistical outliers.

Intra-assay:

# of tests	Mean ng/mL	%CV	Design Goal Specification
20	9.050	8.9	<15%
20	23.084	4.3	<10%
20	70.082	4.1	<10%

Inter-assay:

# of assays	Mean ng/mL	%CV	Design Goal Specification
10	10.179	9.6	<20%
10	25.083	9.5	<12%
10	70.082	4.2	<12%

Dilutional Linearity

In order for any ELISA to give accurate results there must be an excess of antibody (both capture and conjugated) relative to the analyte being detected. It is only under the conditions of antibody excess that the dose response curve is positively sloped and the assay provides accurate quantitation. As the concentration of analyte begins to exceed the amount of antibody the dose response curve will flatten and with further increase will paradoxically become negatively sloped in a phenomenon termed “High Dose Hook Effect”. When the possibility exists that samples may have analyte concentrations in excess of the antibody, it is necessary to assay those

samples at several dilutions to establish if they are on the valid, positively sloped region of the curve or on the negatively sloped hook region of the curve. The issue of hook effect in multiple antigen assays such as this HCP ELISA can be somewhat more complex. The dose response curve for an HCP assay should be thought of as the cumulative dose responses of all HCPs individually, with each HCP having its own hook region determined by the concentration of antibody to that particular HCP. Microtiter plate ELISAs are practically and fundamentally limited in the amount of antibody that can be used. It is common in HCP assays for some 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 still fail to show acceptable dilutional recovery/linearity as evidenced by a significant increase in HCP concentration with increasing dilution. This lack of dilutional linearity is actually the result of the hook effect for the subset of analytes in excess over their respective antibodies. Poor dilutional linearity (Hook Effect) is most likely to be encountered in samples early in the purification process. If the purification process is selective for certain HCPs, poor dilutional linearity may be seen in downstream or even the final product samples. Thus, the establishment of dilutional linearity is a most critical experiment in the development and validation of HCP assays. Dilutional linearity studies are performed at a series of dilutions to establish what we term the "minimum required dilution" (MRD) for a given sample type. The MRD is the first dilution at which the dilution adjusted value for the sample in question remains essentially constant upon further dilution. The HCP value to be reported for such samples is the dilution corrected value at or greater than the established MRD. Once an MRD is established for a particular sample type, your SOP should reflect that this sample requires dilution before assay. We define acceptable dilutional linearity as "dilution corrected analyte concentrations that vary no more than 80% to 120% between doubling dilutions". We evaluated the dilutional linearity from throughout the purification process of six different virus, vaccine, or drug substances. A valid MRD was determined for all samples. Typical dilution data on an in-process sample is shown in the table below. Based on this data we conclude the MRD for this sample is 1:400 and that the HCP concentration is 6 µg/mL

Dilutional Linearity Data:

Sample ID	Dilution	Dilution Corrected value	% change from previous dilution	MRD
In-process	1:100	2.9 µg/mL	NA	
"	1:200	4.3 µg/mL	148%	
"	1:400	6.0 µg/mL	139%	1:400
"	1:800	6.2 µg/mL	103%	

Recovery/Matrix Interference

Defined as the ability of the assay method to correctly quantitate known concentrations of HCP in a representative sample matrix, accuracy was evaluated by spiking 100ng/mL of the same HCP preparation used to make standards into upstream, in-process, and final product samples after dilution, to their established MRDs. This critical experiment demonstrates if anything in the

sample in question interferes in accurately measuring HCP concentrations. The % recovery is calculated as the total measured HCP value in the spiked sample divided by the sum of the amount of material spiked plus the contribution from any endogenous HCP at that dilution. Acceptable recovery is defined as 80% to 120%. Recoveries in all samples from 6 different product streams were all within the acceptable limits ranging from 88% to 112% with a mean recovery of 104%.

Hook Capacity

Very high concentrations of HEK 293 cell HCPs were evaluated for the hook effect. At concentrations exceeding 100µg/mL the apparent concentration of HEK 293 cell HCPs may read less than the 200ng/mL standard. Samples yielding signals above the 200ng/mL kit standard or suspected of having concentrations in excess of 100µg/mL or with certain HCPs in excess of the antibody against that HCP (see section on Dilutional Linearity/Parallelism above) should be assayed at more than one dilution.

Reagent Stability

The critical kit reagents, HRP:antibody conjugate, standards, and coated microtiter plates were evaluated for stability at recommended storage conditions and at elevated temperature (room temperature of ~ 25°C & 37°C) for 4 weeks to attempt to accelerate any instability. The reader should appreciate that these reagents as well as the other non-critical kit reagents (TMB substrate, wash solution, and stop solution) are manufactured by the same methods used for the more than 40 other commercially available ELISA kits manufactured by Cygnus Technologies. The history of these kits shows an excellent stability profile supporting kit shelf lives in excess of 18 months from date of manufacture when stored at 2-8°C. Historically, the stabilities of our typical ELISA components are >10 years for the antibody stored frozen, >3 years for coated plates stored at 2-8°C, >2 years for HRP:antibody conjugates stored at 2-8°C, and >5 years for standards stored frozen. Based on the data we see no indication of unique stability problems with any of the HEK 293 cell HCP assay reagents and thus we project that shelf life for a complete kit will be at least 12 months from date of manufacture.

Report Date

This report was generated January 22, 2010.

Company Information

To obtain additional product information contact Cygnus Technologies:

www.cygnustechnologies.com

Cygnus Technologies, Inc.

4701 Southport Supply Rd. SE, Suite 7

Southport, NC 28461 USA

Tel: 910-454-9442

Fax: 910-454-9443

Email: techsupport@cygnustechnologies.com