Vero Cell HCP ELISA Assay

Validation Summary
Catalog # F500

Summary and Explanation

The data summarized below was generated by Cygnus Technologies to establish the performance parameters and validity of this kit to measure Vero cell Host Cell Proteins (HCPs). This data is intended to supplement and not replace specific user generated validation data. Each end user should validate the assay using their samples to ensure that the assay meets their critical analytical criteria. The data in this report is representative of what a laboratory can expect to achieve when following the recommended protocol as provided in the kit insert. Significant differences in these performance parameters may be indicative of problems with reagents, laboratory equipment, or technique and should be investigated before reporting results.

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 described in detail below was used to generate the validation data. Microtiter plate wells are passively coated with affinity purified goat anti-HCP antibody (VC807-AF), blocked and stabilized with Cygnus Product #I044. The assay uses 6 standards ranging in concentration from 0 to 200ng/mL. 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. Labs demonstrating worse precision than indicated in our laboratories should consider assaying in triplicate

It is recommended that all labs using this kit perform a qualification/validation study to include at least the experiments discussed below.

(1) Each user should perform a western blot using the same antibody used in this kit (VC807-AF) to demonstrate that the antibody reacts with the majority of proteins separated by SDS/PAGE. If you have need to detect and identify individual HCPs, we recommend a method superior to 2D WB involving the 2D HPLC fractionation of HCPs followed by ELISA detection.

(2) Each user should perform intra and inter assay precision experiments to establish their procedural proficiency.

(3) Laboratories should also perform dilutional linearity/ parallelism experiments on their actual samples. This experiment is performed on those samples from the purification process that have significant levels of HCPs. Such samples are to be serially diluted by Sample Dluent Buffer, Cat # I028 the approved diluent for this assay or some appropriate diluent previously shown to give acceptable recovery and background signal. When doubly diluted through the analytical range of the assay, the samples should at some dilution point within the analytical range of the assay, yield essentially the same dilution corrected value at each subsequent dilution. We call this dilution the "Minimum Required Dilution" or MRD. This critical experiment establishes the condition of antibody excess for accurate quantitation and determines that typical process samples do not have HCPs in the "Hook Region" of the concentration response curve.

(4) Each user should perform spike recovery experiments using their test sample matrices and actual samples. This experiment will establish the degree of sample matrix interference in the recovery of HCPs. Such a study can be performed by adding known amounts of the 200ng/mL standard provided with the kit to the final product or any intermediate samples to be tested. Ideally these test sample matrices should be devoid of any Vero cell proteins or have very low levels (<8ng/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-Vero cell:HRP Conjugate, Lots 1338-38 & 1338-48	Cat #F501
Microtiter coated plate, Lots 538, 10038, 1338	Cat #F502
Vero cell HCP Standards, Lots 3138 & 248	Cat #F503
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.	#Vero cell 1-07, Pages 1-25
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 Vero cells extracted by a procedure similar to what is used to harvest virus. The antibody was western blotted after 2 Dimensional separation of the HCPs using PAGE and Isoelectric focusing. Western blot reactive areas were compared to a duplicate 2D silver stained gel. Within the limits of these 2 orthogonal methods, it was concluded the antibodies recognize the majority of Vero cell HCPs. Four cell lines provided by various companies using Vero cells for expression of various vaccine products were evaluated. All 4 strains showed evidence of conservation among the vast majority of HCPs. Thus, this kit should be of utility for strains other than those specifically used above. It is recommended that each user of this kit verify by western blotting and ELISA that the kit antibody reacts with the majority of their HCPs.

Assay Standardization/Calibration

Vero HCPs from a non-virus transfected cell line were extracted following methods similar to initial steps used to recover crude product. The resulting extract was sterile filtered at 0.2 μ and diafiltered against a 3000 molecular weight cutoff membrane. The resulting HCP reference preparation was assigned a total Vero cell HCP concentration of 3.1mg/mL using the BCA protein assay with BSA as the standard.

Standard Curve

Typical standard curve data from an actual assay run using a point to point fit is shown below. Do not use linear regression to fit the curve and interpolate sample values! 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 R^2, 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	0.112 0.109	0.111	1.9
2ng/mL	0.142 0.141	0.142	0.5
8ng/mL	0.205 0.208	0.207	1.0
25ng/mL	0.400 0.421	0.411	3.6
75ng/mL	0.957 1.013	0.985	4.0
200ng/mL	2.299 2.316	2.308	0.5

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	7.49	6.9	<20%
20	23.16	5.5	<10%
20	75.32	3.5	<10%

Inter-asay:

# of assays	Mean ng/mL	%CV	Design Goal Specification
10	8.06	4.4	<20%
10	24.57	3.7	<10%
10	73.95	3.6	<10%

Sensitivity

Limit of Detection (LOD) - The Vero 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 700pg/mL.

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 for the assay at several low concentration points and then interpolating that concentration which corresponds to a 20% CV. The %CV for 20 replicates of the 2ng/mL standard was 16.8%. This data suggests an LOQ of ~1ng/mL can be statistically obtained. Because the lowest dosed standard for the assay is 2ng/mL and that precision can be variable from lab to lab and technician to technician, we conservatively claim the LOQ as <2ng/mL.

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 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 4 samples for dilutional linearity from various points in the purification processes of a viral vaccine product. Once samples were diluted within the analytical range of the assay only sample #3 demonstrated a lack of dilutional linearity. With greater dilution an MRD of 1:64 was demonstrated for this sample with the value reported as 593 ng/mL.

Dilutional Linearity Data:

Sample ID	Dilution	Dilution Corrected Value	% change from previous dilution	MRD
#1 Bulk Harvest	1:100	17.4µg/mL	NA	1:100
"	1:400	17.7µg/mL	102%	
"	1:1600	17.8µg/mL	101%	
#2 Sucrose Gradient	1:100	4.47µg/mL	NA	1:100
"	1:400	4.53µg/mL	101%	
"	1:1600	4.29µg/mL	95%	
"	1:6400	3.60µg/mL*	84%	
#3 Post SEC	neat	219ng/mL	NA	
"	1:4	440ng/mL	201%	
"	1:16	530ng/mL	120%	
	1:64	593ng/mL	112%	1:64
	1:128	574ng/mL	97%	
"	1:256	583ng/mL	102%	
#4 Drug Substance	neat	79ng/mL	NA	neat
"	1:2	79ng/mL	100%	
"	1:4	74ng/mL	95%	

* The HCP value prior to dilution correction was less than the LOQ of the method at ~2ng/mL. Values the LOQ are considered statistically unreliable and should not be reported.

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 an in-process and 3 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%.

% Spike & Recovery at 100ng/mL HCP of Vaccine Samples:

Sample ID	MRD	Endogenous Vero HCP Concentration at MRD	Total HCP concentration measured (endogenous + 100ng/mL spike)	% Recovery Total HCP detected/ (endogenous +100ng/mL)
#1	1:64	9.27ng/mL	95.5ng/mL	87.4
#2	Neat	3.55ng/mL	97.27ng/mL	93.9
#3	Neat	<2ng/mL	102.9ng/mL	102.9
#4	Neat	2.46ng/mL	100.85ng/mL	98.4

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 28 days 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. For

standards, the relative activity is defined as that obtained for standards stored frozen at -80°C and only thawed one time. Based on the data summarized below, we see no indication of unique stability problems with any of the Vero 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 when stored at 2-8°C. Our SOPs only allow for extensions of expiration dates based on real time and temperature storage conditions.

Accelerated Stability Data on Critical Assay Components:

Component	Lot #	Storage Conditions	Age at Testing	% change in Activity
Standards	248	-80°C	28 days	control
"	"	-20°C	28 days	100.5%
"		4°C	28 days	100.5%
"	"	Room Temp. ~27°C	28 days	96.5%
"	"	37°C	28 days	86.7%
HRP Conjugate	1338-48	2-8°C	28 days	control
"	"	Room Temp. ~27°C	28 days	ODs 2.25% lower
"	"	37°C	28 days	ODs 1.1% lower
Coated Plates	538	2-8°C, with desiccant	28 days	control
"	"	Room Temp. ~27°C without desiccant	28 days	105%

Hook Capacity

Very high concentrations of Vero cell HCPs were evaluated for the hook effect. At concentrations exceeding 1mg/mL the apparent concentration of Vero cell HCPs may read less than the 200ng/mL kit standard. Samples yielding signals above the 200ng/mL standard or suspected of having concentrations in excess of 1mg/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. While an MRD can be established as a result of your validation study, we suggest assaying all samples using at least 2 dilutions around the MRD, until your batch-to-batch process control has been established.

Report Date

This report was generated April 21, 2008.

Company Information

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