

# CHO HCP ELISA Assay, 3G

## Validation Summary Catalog # F550

### Summary and Explanation

The data summarized below was generated by Cygnus Technologies to establish the performance parameters and validity of this kit to measure CHO 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.

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 study to determine antibody reactivity to individual HCPs from their cell line and process. Western blot provides limited qualitative data in this regard. 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 the approved diluent for this assay, Cat # 1028 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 100ng/mL standard provided with the kit to the final product or any intermediate samples to be tested. Samples containing endogenous, process derived HCP should be diluted at least to their MRD as established in (3) above prior to making the spikes. If the level of endogenous HCP is high relative to the intended spike levels

such samples may need to be diluted below the MRD to better determine spike recovery.

### Materials & Methods Used

Materials	
Goat anti-CHO:HRP Conjugate, Lots 89-89, 89-99, and 89-119	Cat #F551
Microtiter coated plate, Lots 189,11089, 2199	Cat #F552
CHO HCP Standards, Lot 789	Cat #F553
The protocol as defined in the kit insert was used in this validation.	
<b>Data References:</b> Raw data for these experiments are recorded in Cygnus Notebook.	<b>#2009, Pages 1-31</b>
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

Our analysis and qualification of samples from 5 commercial products expressed in CHO cells indicates that most of the proteins are conserved among all cell lines. That data suggests this assay should be useful for detecting HCPs from other CHO derived products. Historically 1 & 2 dimensional Western blot have 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. Western blot has poor sensitivity and specificity relative to ELISA. As such, 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. Similarly, the presence of a Western blot band or spot does not assure that the ELISA will be able to detect that particular protein. For these reasons, we now use and recommend a method far superior to 2D Western blot in determining the polyclonal antibody reactivity to individual HCPs. This method involves the fractionation of HCPs by 2 dimensional liquid chromatography (2D HPLC). This involves a chromatofocusing fractionation in the first dimension followed by "Reversed Phase" gradient elution chromatography in the second dimension. This method yields highly purified, individual liquid phase HCPs in a more native, non-denatured configuration. This method allows

for the collection of hundreds to thousands of separate fractions that can then be semi-quantitatively analyzed in the much more sensitive and specific ELISA with greater ease and objectivity than can be obtained by 2D WB. This method can detect individual HCPs in even downstream samples where the product protein is too high to allow for detection by 2D WB. We performed the 2D HPLC fractionation on conditioned media from two CHO strains, CHO-S and a K1 strain. Using our 2D HPLC-ELISA method we were able to detect 791 antibody reactive individual fractions/proteins in CHO-S conditioned media and 770 reactive fractions from the K1 strain. These 770+ proteins represent greater than 98% of the total protein as detected by OD214 in the fractions eluting from the Reversed Phase column. In addition, we performed 2D HPLC fractionation of a mild lysate of CHO-S cell and detected antibody reactivity in 658 fractions. A downstream-purified drug substance containing about 200ppm of "total HCP" was also fractionated by 2D HPLC. This ELISA detected HCP activity in 128 separate fractions. The above data is offered as a qualitative indication that we expect would be seen in any CHO strain grown under typical culture conditions. This data is not intended to replace experiments that may be required to qualify this antibody specifically for your strain. Should you desire to evaluate the reactivity of this antibody to your strain HCPs Cygnus is pleased to offer a service and/or consultation for fractionation of HCPs using 2 Dimensional HPLC methods followed by detection in the ELISA.

## 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 described in detail below was used to generate the validation data. Microtiter plate wells are passively coated with affinity purified goat anti-HCP antibody, blocked and stabilized. The assay uses 6 standards ranging in concentration from 0 to 100ng/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 in the coated well. The effects of sample volume, incubation times, and antibody conjugate concentration were also evaluated in selecting the final protocol.

Analysis of these variables indicates that the assay and its antibodies are robust and 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. The validation study was completed using a simultaneous assay protocol as summarized below with duplicate analysis of all standards, controls and samples. Labs demonstrating worse precision than indicated in our laboratories (in the range of 8%CV or higher) should consider assaying in triplicate.

CHO HCPs obtained from conditioned media were for used as the source material for assay standards/calibrators. After further processing to remove non-HCP components involving UF/DF,

the resulting HCP reference preparation was assigned a total CHO HCP concentration of 20µg/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. 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<sup>2</sup>, 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	0.140 0.153	0.147	6.3
1ng/mL	0.186 0.182	0.184	1.5
3ng/mL	0.239 0.239	0.239	0
12ng/mL	0.517 0.503	0.510	1.9
40ng/mL	1.333 1.333	1.333	0
100ng/mL	2.539 2.565	2.552	0.7

## 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	2.95	6.9	<15%
20	11.81	2.7	<10%
20	53.80	3.7	<10%

Inter-assay:

# of assays	Mean ng/mL	%CV	Design Goal Specification
12	3.00	7.2	<15%
12	11.87	4.4	<10%
12	63.03	5.0	<10%

## Sensitivity

Limit of Detection (LOD) - The CHO 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 and the lowest standard at 1ng/mL. The mean signal of the zero standard plus 2SD yielded a LOD of 0.3ng/mL as interpolated from the point to point plot of the mean ODs for the zero and 1ng/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 0.25ng/mL and 1ng/mL. The %CV for 20 replicates of the 1ng/mL control was 19.6%. The %CV for 20 replicates of the 0.25ng/mL control was 53.5%. This data suggests an LOQ of ~1ng/mL can be obtained.

## 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 4 samples for dilutional linearity from 4 steps in a purification process of a CHO cell expressed product. A valid MRD could be determined for all 4 samples. Typical dilution data is shown below.

Dilutional Linearity Data:

Sample ID	Dilution	Dilution Corrected value	% change from previous dilution	MRD
#1 1 <sup>st</sup> purification step	1:20	1.74µg/mL	NA	1:20
"	1:40	1.90µg/mL	109%	
"	1:80	2.00µg/mL	105%	
"	1:8000	2.31µg/mL	115%	
#2 2 <sup>nd</sup> purification step	1:2	54ng/mL	NA	
"	1:4	62ng/mL	115%	
"	1:8	68ng/mL	110%	1:8
"	1:16	70ng/mL	103%	
#3 3 <sup>rd</sup> purification step	neat	34ng/mL	NA	
"	1:2	44ng/mL	129%	
"	1:4	49ng/mL	111%	1:4
"	1:8	58ng/mL	118%	
#4 Final Drug Product	neat	1.9ng/mL	NA	
"	1:2	2.5ng/mL	132%	1:2
"	1:4	2.3ng/mL	92%	
"	1:400	2.2ng/mL	96%	

## 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 50ng/mL of the same HCP preparation used to make standards into various in-process buffer matrices as well as in-process and final product samples after dilution, to or below 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 were all within the acceptable limits ranging from 92% to 115%.

## 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 summarized below, we see no indication of unique stability problems with any of the CHO 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	789	-80°C	4 weeks	control
"	"	2-8°C	4 weeks	<2%
"	"	Room Temp. -27°C	4 weeks	~6%
"	"	37°C	4 weeks	~13%
HRP Conjugate	89-89	2-8°C	4 weeks	control
"	"	Room Temp. -27°C	4 weeks	~18%
"	"	37°C	4 weeks	~20%
Coated Plates	189	2-8°C, with desiccant	4 weeks	control
"	"	Room Temp. -27°C without desiccant	4 weeks	~11%

## Hook Capacity

Very high concentrations of CHO HCPs were evaluated for the hook effect. At concentrations exceeding 1mg/mL the apparent concentration of CHO HCPs may read less than the 100ng/mL kit standard. Samples yielding signals above the 100ng/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 August 22, 2009.

## Company Information

To obtain additional product information contact Cygnus Technologies:

[www.cygnustechnologies.com](http://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](mailto:techsupport@cygnustechnologies.com)