The lack of endogenous HCP is high relative to the intended spike levels established in (3) above prior to making the spikes. If the level of derived HCP should be diluted at least to their MRD as samples to be tested. Samples containing provided with the kit to the final product or any intermediate performed by adding known amounts of the interference in the recovery of HCPs. Su experiment will establish the degree of sample matrix using their test sample matrices and actual samples. This each user should perform spike recovery experiments 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. 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

<table>
<thead>
<tr>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-A549:HRP Conjugate, affinity purified antibody from a pool of 2 goats</td>
</tr>
<tr>
<td>Microtiter coated plates, coated with a blend of affinity purified antibody from the same 2 goats</td>
</tr>
<tr>
<td>A549 HCP Standards, mild lysate of null A549 cells</td>
</tr>
<tr>
<td>The protocol as defined in the kit insert was used in this validation.</td>
</tr>
</tbody>
</table>

Data References: Raw data for these experiments are recorded in Cygnus Notebook #1-A549.

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 commercial products expressed in A549 cells indicates that most of the proteins are conserved among all cell lines and culture processes. That data suggests this assay should be useful for detecting HCPs from other A549 derived products. Historically 1 & 2 dimensional WB have been used to characterize anti-HCP antibodies. Because of the limitations for both sensitivity and specificity, WB is of little to no predictive value in determining how the ELISA quantitatively reacts to the more limited array of HCP in downstream and final product samples. The lack of identity between silver stain and western blot does not necessarily mean there is no 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 detect the ELISA. For these reasons Cygnus has only characterized the kit antibodies by 1D WB showing broad reactivity to all major HCPs. Should you desire to evaluate the reactivity of this antibody to individual HCPs from your strain, Cygnus is pleased to offer 2D WB or 2 Dimensional HPLC-ELISA analysis.

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. Microwell 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 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 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.

A549 HCPs obtained from a mild lysate were for use 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 A549 HCP concentration 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 R², slope, intercept, upper and lower asymptotes etc. are too indirect and insensitive to provide critical analytical control.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Duplicate OD Values</th>
<th>Mean OD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0ng/mL</td>
<td>0.051, 0.049</td>
<td>0.050</td>
<td>2.8</td>
</tr>
<tr>
<td>2ng/mL</td>
<td>0.064, 0.065</td>
<td>0.065</td>
<td>1.1</td>
</tr>
<tr>
<td>8ng/mL</td>
<td>0.114, 0.111</td>
<td>0.239</td>
<td>1.9</td>
</tr>
<tr>
<td>25ng/mL</td>
<td>0.250, 0.246</td>
<td>0.248</td>
<td>1.1</td>
</tr>
<tr>
<td>75ng/mL</td>
<td>0.587, 0.642</td>
<td>0.615</td>
<td>6.3</td>
</tr>
<tr>
<td>200ng/mL</td>
<td>1.595, 1.674</td>
<td>1.634</td>
<td>3.4</td>
</tr>
</tbody>
</table>

### 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:

<table>
<thead>
<tr>
<th># of tests</th>
<th>Mean ng/mL</th>
<th>%CV</th>
<th>Design Goal Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>8.6</td>
<td>12.0</td>
<td>&lt;20%</td>
</tr>
<tr>
<td>10</td>
<td>27.1</td>
<td>2.9</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>10</td>
<td>194</td>
<td>4.9</td>
<td>&lt;10%</td>
</tr>
</tbody>
</table>

#### Inter-assay:

<table>
<thead>
<tr>
<th># of assays</th>
<th>Mean ng/mL</th>
<th>%CV</th>
<th>Design Goal Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>9.1</td>
<td>7.4</td>
<td>&lt;15%</td>
</tr>
<tr>
<td>5</td>
<td>24.9</td>
<td>4.4</td>
<td>&lt;15%</td>
</tr>
<tr>
<td>5</td>
<td>74.1</td>
<td>6.5</td>
<td>&lt;15%</td>
</tr>
</tbody>
</table>

### Sensitivity

Limit of Detection (LOD) - The A549 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 0.5ng/mL as interpolated from the point to point plot of the mean ODs for the zero and 2ng/mL standards.

Lower Limit of Quantitation (LLOQ) - LLOQ 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 1 ng/mL and 2ng/mL. This data suggests an LLOQ of ~1.6 ng/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 doubling 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 2 final product (virus) samples for dilutional linearity. A valid MRD could be determined for both samples.

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 A549 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.

<table>
<thead>
<tr>
<th>Component</th>
<th>Storage Conditions</th>
<th>Age at Testing</th>
<th>% Change in Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td>-80°C</td>
<td>4 weeks</td>
<td>control</td>
</tr>
<tr>
<td></td>
<td>2-8°C</td>
<td>4 weeks</td>
<td>&lt;2%</td>
</tr>
<tr>
<td></td>
<td>Room Temp. (~25°C)</td>
<td>4 weeks</td>
<td>~6%</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>4 weeks</td>
<td>~13%</td>
</tr>
<tr>
<td>HRP Conjugate</td>
<td>2-8°C</td>
<td>4 weeks</td>
<td>control</td>
</tr>
<tr>
<td></td>
<td>Room Temp. (~25°C)</td>
<td>4 weeks</td>
<td>~18%</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>4 weeks</td>
<td>~20%</td>
</tr>
<tr>
<td>Coated Plates</td>
<td>2-8°C, with desiccant</td>
<td>4 weeks</td>
<td>control</td>
</tr>
<tr>
<td></td>
<td>Room Temp. ~25°C, without desiccant</td>
<td>4 weeks</td>
<td>~11%</td>
</tr>
</tbody>
</table>

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 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%.

Hook Capacity

Very high concentrations of A549 HCPs were evaluated for the hook effect. At concentrations exceeding 0.2mg/mL the apparent concentration of A549 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 0.2mg/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.
Company Information

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