

## Validation of HCP & Bioprocess Contaminant Assays

This study protocol is suggested as an objective method to validate that the *Cygnus Technologies* Host Cell Protein (HCP) ELISA kits and other bioprocess contaminate kits will yield accurate results for a given product and sample type. In addition to the ELISA validation protocol detailed below, it may also prove helpful to characterize the antibodies used in the HCP ELISA kits by Western blot analysis against your process specific HCPs. There are fundamental differences between Western blot and ELISA in terms of specificity and sensitivity that should be appreciated. First, ELISA is a much more sensitive procedure than Western blot yielding detection limits more than 100 fold lower. As such, ELISA is typically the only method with adequate sensitivity to detect contaminants in downstream and final product. ELISA in general can be much more specific than Western blot and does not give false positive, non-specific reactions as are encountered in many Western blot procedures. Western blot has an important advantage over ELISA in that it can identify individual HCPs whereas ELISA cannot differentiate one HCP from another. For this reason it is advisable to characterize the antibody used in the ELISA by Western blot. This is typically a one time experiment with the goal of demonstrating that the majority of protein bands separated by PAGE analysis (either one or 2 dimensional) and stained by a non-specific protein staining method such as Coomassie, silver stain, or colloidal gold stain, will have corresponding antibody reactive western blot bands. Because of the poor relative sensitivity of western blot versus ELISA, the western blot experiment should only be performed on starting material such as clarified culture media or in some cases on samples after an additional purification step provided the ratio HCP to product is well above the part per thousand range. Interpretation of western blot and PAGE bands can be a subjective endeavor and thus it may prove necessary to resolve any differences between the western blot and PAGE protein stain by further carefully controlled experimentation. Users of our kits and antibodies are encouraged to contact our Technical Services for help in interpretation and design of western blot experiments.

In some cases your product itself or certain components in product formulation buffer may interfere (either positive or negative interference) in the ability of the assay to detect HCPs or other contaminants. Similarly, samples from upstream in the purification process may also contain material in their matrices that can interfere in ELISA methods. Factors such as extremes in pH, detergents, organic solvents, high protein concentration, and high buffer salt concentrations are known interference components. For these reasons it is necessary to validate by universally recognized experimental procedures (i.e. ICH & FDA guidelines) that the assay will yield accurate results. Should the end user of this kit determine that there is significant product or matrix interference it may be necessary to further process the sample by methods such as dilution or buffer exchange to render it into a more assay compatible buffer. The same diluent used to prepare the kit standards is ideally the preferred material for dilution or buffer exchange of your samples. In other cases, modification of the assay protocol can affect

improved accuracy in some sample types. Users of our kits are encouraged to contact the Technical Services Department for advice on how best to solve sample accuracy issues.

**1. Spike and recovery experiments** - For each sample type to be tested, be it final product or in-process samples, you should demonstrate that the assay can recover added HCP or other contaminant spiked into that sample matrix. This can be simply performed by spiking the highest standard provided with the kit into your sample types and then testing in the assay. Using the *E. coli* HCP kit, Cat # F410 as an example, we suggest spiking 1 part of the 100ng/mL standard into 4 parts of your sample (e.g. spike 100 $\mu$ L of 100ng/mL standard into 400 $\mu$ L of sample). The spiked concentration into the sample in this case is 20 ng/mL. A control dilution of 1 part of assay diluent (zero standard) to 4 parts of sample is also performed to determine the contribution of endogenous HCP in the sample prior to spiking. Both the spiked and diluted-unspiked sample are assayed. Percent added recovery is determined by subtracting the endogenous contribution of HCP from the total HCP measured in the spiked sample. We suggest acceptable recovery should be within 80% to 120% of the spiked HCP. Table 1 shows example data. If you desire spike and recovery at more than one concentration we recommend that the lowest spike levels should be at least 2 times the Limit of Quantitation (LOQ) of the assay and that the contribution of the endogenous HCP in the sample prior to spiking not exceed two times the spike level to be tested. These two conditions will insure better statistical accuracy.

**Table 1**  
**Example Spike and Recovery Data**

Sample	Spike Conc. (ng/mL)	Total HCP measured (ng/mL)	% Spike Recovery
4 parts final product + 1 part zero standard	0	3	NA
4 parts final product + 1 part 100ng/mL std.	20	22	95% [(22-3)/20]

**2. Dilutional linearity/recovery experiments** – Sample types with levels of contaminant greater than the LOQ of the assay should initially be evaluated for dilutional linearity as part of assay validation. This experiment involves performing a number of serial dilutions using an approved assay diluent. These dilutions are then assayed and a dilution corrected contaminant concentration is determined at each dilution. This dilutional linearity study establishes freedom of sample matrix interference as well as the important condition of antibody excess for the array of contaminants in your samples. If you will be routinely testing in-process samples

in addition to final product, you should validate dilutional linearity of each sample type. This analysis is critical for HCP assays because very high concentrations of certain HCPs may approach saturation of the antibody against that particular HCP. When this happens there is a risk of under-quantitation for that HCP. By performing dilutional analysis one can verify if the antibody is in excess and that the sample matrix itself does not interfere. If the antibody is in a limiting concentration or the sample matrix causes a negative interference what will be observed is that the apparent HCP concentration for a sample increases with increasing dilution. In most cases a dilution will be reached where the dilution corrected value remains essentially constant. This dilution is what we term the Minimum Required Dilution or MRD. Table 2 below shows example data where a sample did not yield good dilutional linearity at high concentration but with further dilution an MRD was determined at which acceptable dilutional linearity was obtained. In this example we conclude that the MRD for this in-process sample is 1:8 and that the concentration of HCP to be reported is 361ng/mL. Once an MRD is established for a particular sample type, your SOP should reflect that such a sample needs to be diluted before assay. We suggest defining acceptable dilutional linearity as “dilution corrected analyte concentrations that vary no more than 80% to 120% between doubling dilutions”. Due to the statistical limitations in the low end of the assay range you should avoid consideration of dilutional data where the assay value before dilution correction falls below two times the LOQ of the assay. Acceptable diluents may vary from assay to assay and you are encouraged to verify with *Cygnus* that your sample diluent is acceptable. In general, the best diluent is the same one used to prepare the kit standards. Assay specific diluents can be purchased in 100ml, 500ml or 1L bottles.

Table 2

Example Dilutional Linearity Data for an In-Process Sample

Sample Dilution	Dilution corrected value (ng/mL)	% Change in concentration from previous dilution
Neat (undiluted)	146	NA
1:2	233	160%
1:4	312	134%
1:8	361	116%
1:16	356	99%
1:32	370	104%
1:64	Not calculated ( $< 2$ times LOQ)	NA

**3. Precision Experiments** - We also recommend that each laboratory and perhaps each technician perform a precision study to demonstrate that they can achieve acceptable precision both within and between runs. Such precision would be best accomplished on controls prepared in your laboratory. Those controls should be made in your product matrix, aliquoted and stored frozen to insure stability. Once statistical values have been established on your controls you will then have an important QC tool to assure that the accuracy is acceptable from kit to kit and assay run to assay run.