Poor Dilution Linearity

Demonstration of dilution linearity (also termed dilution parallelism or dilution recovery) of samples containing the analyte of interest is a critical experiment to validate the specificity and accuracy of a given method. Assays for multiple analytes such as our HCP ELISA will often show a lack of dilution linearity for certain samples. In the case of HCP assays this lack of dilution linearity is usually due to insufficient excess of antibody for some of the HCPs found in your sample. Other causes for poor dilution linearity are due the product protein itself or certain components in product formulation buffer that may interfere (either positive or negative interference) in the ability of the assay to detect HCPs or other contaminants. Similarly, samples from various points 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 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 improve accuracy in some sample types. Users of our kits are encouraged to contact Technical Services Department for advice on how best to solve sample accuracy issues.

Sample types with levels of contaminants greater than the LOQ of the assay should, as part of assay validation, initially be evaluated for dilution linearity. This experiment involves performing a number of serial dilutions using an approved assay diluent. These dilutions are then assayed, and a dilution corrected HCP concentration is determined at each dilution. This dilution linearity study is particularly important for a HCP assay because it establishes the important condition of antibody excess for the array of HCPs in your samples. If you will be routinely testing in-process samples in addition to final product, each sample type should have a dilution linearity validation. This analysis is necessary 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 dilution 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, you will observe 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 1 below shows example data where a sample did not yield good dilution linearity at a 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 this sample needs to be diluted before assay. Your samples from a given point in the purification process could vary somewhat from lot to lot. For this reason, we suggest assaying samples at 3 doubling dilutions; one at the MRD, one above, and one below, until your process reproducibility and control can established. We suggest defining acceptable dilution 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 dilution 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 Technologies 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 from Cygnus in 100ml, 500ml or 1000mL bottles. Contact Cygnus for information on acceptable diluents.

Table 1. Example of Dilution Linearity Data for an In-Process Sample

Sample Dilution	Dilution corrected value (ng/mL)	% change in concentration from previous dilution
Neat (undiluted)	146	N/A
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)	N/A