KRISHGEN BioSystems
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VALIDATION OF KRIBIOLISA CHO HCP ELISA KIT AS PER ICH Q2(R1) METHODOLOGY & ANALYTICAL PROCEDURES GUIDELINES

This validation protocol has been adopted in line with the Methodology and Analytical Procedures Guideline Q2(R1) developed by ICH Expert Working Group.

Document History

First Codification	History	Date

Version#1	**VALIDATION DATA OF KRIBIOLISA CHO HCP ELISA (Cat No#KBBP03)**	1st Oct, 2010
Version#2	**Approved by QC and renamed as per ICH guidelines Q2(R1)**	27th Mar, 2011
Version#3	**Approved by QC and renamed as per ICH guidelines Q2(R1)**	14th Jan, 2012

Approved Quality Control	Approved Product Development	Approved Operations Head
14.01.2012	14.01.2012	14.01.2012

1. Introduction

This document presents a discussion of the characteristics of our KRIBIOLISA CHO ELISA kit considered by us during the validation of this kit in accordance with ICH Q2(R1) guidelines. The document is prepared based on tests run in our laboratory and does not necessarily seek to cover the testing that may be required at user's end for registration in, or regulatory submissions. The objective of this validation is to demonstrate that it is suitable for its intended purpose – determination of host cell proteins in CHO cell culture.

Validation characteristics considered by us in accordance with the guidelines are listed below :

- **Accuracy**
- **Quantitation Limit**
- **Detection Limit**
- **Precision**
  - **Repeatability**
  - **Intermediate Precision**
- **Robustness**
- **Specificity**
- **Linearity**
- **Range**

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

For any queries or support on the data and its performance, please contact us at sales@krishgen.com

1. Sensitivity:

a) **Limit Of Detection:** It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was found to be 0.6ng/ml.
b) **Limit of Quantitation:** It is defined as the lowest concentration for which Coefficient of Variation is <20%. The LOQ is found to be <1 ng/ml.

2. Specificity / Cross reactivity:

Antibodies used in the kit have been tested for reactivity by Western Blotting against several strains of CHO cells (CHO-S, CHO-K1) indicating that most of the proteins are conserved among all strains.

3. Precision:

Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (5 ng/ml) and high (50 ng/ml) concentrations. 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.

Pool	Intra Assay %CV	Inter Assay %CV
Low	9.03%	4.76%
High	9.58%	13.69%

4. Recovery by Spiking:

In spike and recovery, a known amount of analyte is added (spiked) into the natural test sample matrix and its response is measured (recovered) in the assay by comparison to an identical spike in the standard diluents.

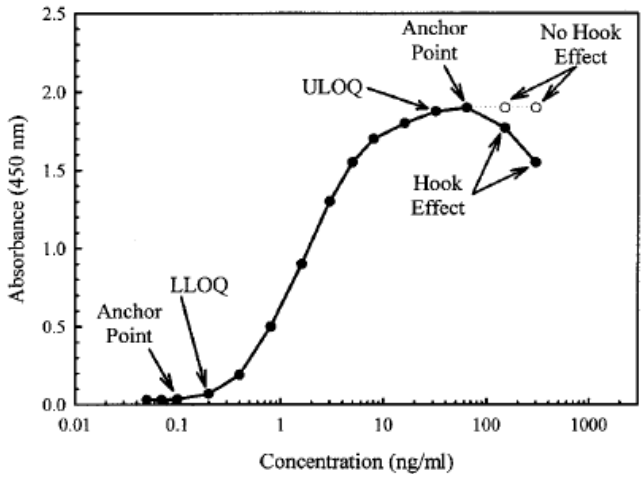
Commonly used Buffer	HCP Added (ng/ml)	Expected	Observed	Recovery (%)
Buffer 1	5	5	4.5	90%
	50	50	57.5	115%
	100	100	110	110%
Buffer 2	5	5	5.2	104%
	50	50	48	96%
	100	100	110	110%
Buffer 3	5	5	4.9	98%
	50	50	55	110%
	100	100	120	120%

5. Standard Curve Characteristics:

Correlation Coefficient (r)	Intercept (A)	Slope (B)
-0.999	**2.867**	**-1.850**

6. High Dose Hook Effect:

The high dose hook effect refers to measured levels of antigen displaying a significantly lower absorbance than the actual level present in a sample. This appears when a simultaneous ELISA assay is saturated by a very high concentration of sample antigen binding to all available sites on both the solid phase antibody as well as the detection antibody and thereby preventing the sandwich-formation. The antigen-saturated detection antibodies in solution will be washed off giving a falsely low signal. A "hook" is observed in the curve when data is plotted as a signal versus antigen concentration.



A high dose hook is indicated in the plotted curve when the assay is saturated by high antigen concentrations.

Increasing concentrations of HCPs >250 ng/ml were assayed as unknowns. **The hook capacity yielding an absorbance reading less than the 250 ng/ml standard was ~0.1 mg/ml.**

7. Dilutional Linearity:

Dilutional linearity should be evaluated on spikes that have been made into the sample matrix and then are diluted into the assay matrix. The matrix can comprise individual or a pool of individual samples. The choice of pooled vs. individual samples depends on whether interference from substances may be of concern. Dilutions should be made such that several dilutions fall on the standard curve.

Evaluation of dilutional linearity should be done with a spike made 100- fold greater than the ULOQ. The samples should also include a dilution above the ULOQ (to evaluate the hook curve effect). It is common practice that individual dilution step should not exceed 1:100.

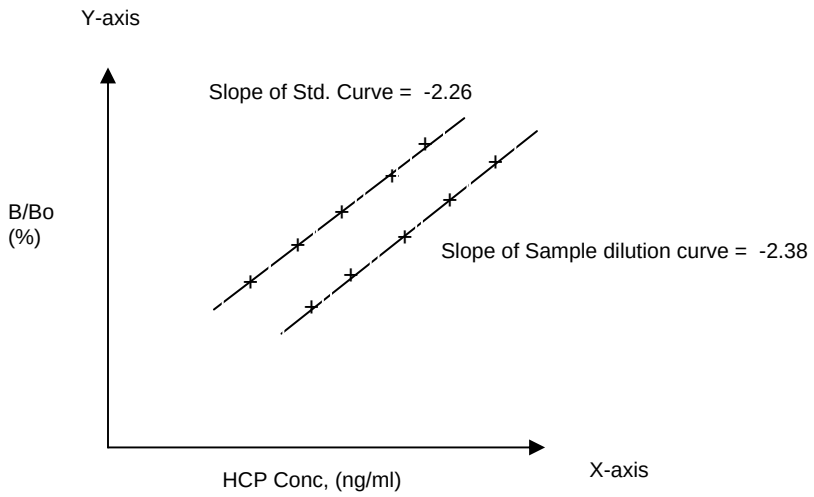
When there is lack of dilutional linearity an appropriate strategy for analysis of high concentration samples must be established. The use of MRD (Minimum Required Dilution) or a Plateau Value before reporting may suffice this need.

Typical dilution data for MRD is indicated herein below:

Sample ID	Dilution	Absorbance		Mean	B/Bo	Conc. from graph ng/ml	Conc. (X with DF) ng/ml	% Change in Conc. from previous dilution	MRD
		1	2						
Sample 1 (Post Cell Harvest)	1:800	2.049	2.099	2.074	20.570	230.00	184000	NA	1 : 6400
	1:1600	1.563	1.556	1.560	45.413	69.50	111200	60.435	
	1:3200	1.118	1.123	1.121	66.610	27.50	88000	79.137	
	1:6400	0.839	0.849	0.844	79.961	14.50	92800	105.455	
Sample 2 (Prior to Ultrafiltration)	1:400	2.06	2.038	2.049	21.777	230.50	92200	NA	1 : 800
	1:800	1.821	1.867	1.844	31.676	112.20	89760	97.354	
	1:1600	1.417	1.546	1.482	49.179	59.00	94400	105.169	
	1:3200	1.167	1.195	1.181	63.689	32.00	102400	108.475	
Sample 3 (Post Anion Exchange Chromatography)	1:400	1.646	1.646	1.646	41.200	80.00	32000	NA	1 : 800
	1:800	1.239	1.239	1.239	60.800	39.00	31200	97.500	
	1:1600	0.915	0.93	0.923	75.000	20.00	32000	102.564	
	1:3200	0.738	0.742	0.740	85.500	9.50	30400	95.000	
Sample 4 (Post Protein Purification)	1:200	1.797	1.817	1.807	33.462	111.20	22240	NA	1 : 400
	1:400	1.492	1.444	1.468	49.831	58.00	23200	104.317	
	1:800	1.174	1.114	1.144	67.890	28.50	22800	98.276	
	1:1600	0.852	0.858	0.855	79.430	14.50	23200	101.754	

8. Parallelism:

Parallelism is assessed with multiple dilutions of samples that represent the same matrix and analyte combination. We have assayed the standard line along with the sample in linear dilution. The data given below shows both the standard line and the sample line to be parallel to each other with the slope being similar indicating sample-matrix has no substantial influence on sample estimates.



Standard Curve

Std. (ng/ml)	Abs1	Abs2	Mean Abs.	B/Bo %
0	0.418	0.440	0.429	100.00
1	0.486	0.486	0.486	97.20
4	0.567	0.560	0.564	93.50
20	0.913	0.933	0.923	76.14
75	1.440	1.464	1.452	50.60
250	2.148	2.163	2.156	16.90

Slope of Standard Curve = - 2.26

Sample Dilution	Abs1	Abs2	Mean Abs.	B/Bo %	Conc. (X with DF) ng/ml
1:200	1.797	1.817	1.807	33.46	22240.00
1:400	1.492	1.444	1.468	49.83	23200.00
1:800	1.074	1.114	1.094	67.89	21600.00
1:1600	0.852	0.858	0.855	79.43	23200.00

Slope of Sample Dilution curve = - 2.38

9. Interpretation of Data:

Queries have been raised by our users on the exact methodology to be followed for interpretation of the data generated in terms of the absorbance of the unknown samples and known standards of the assays. Of the methods surveyed the logistic-log and fully specified logit-log functions are the most accurate models for forming standard curves and for interpolating HCP concentrations from the standard curve. The accuracy of the fully specified logit-log function is highly dependent on the precise specification of two unknown quantities, the optical densities at zero and infinite concentrations, prior to fitting the model to a typical set of calibration data.

The function does not require pre-specification of any parameters before estimating the standard curve, and the four parameters are readily interpretable in terms of identifiable physical quantities. This model also has the advantage that it is easiest to visualize since it does not incorporate complex transformations of the optical density scale.

Hence we have adopted the use of data reduction program (logit-log) to determine the HCP concentration in the unknown samples.

Plot the % bound on the vertical axis (logit) against the HCP concentrations on the horizontal axis (log) for each standard (except the zero standard). Alternatively, calculate percent conjugate bound (%B) for each standard, and sample relative to the maximum binding (B_0, Zero standard) wells as follows:

$$\%B/B_0 = \frac{\text{Mean Absorbance (highest standard) - Mean Absorbance (standard/sample)}}{\text{Mean Absorbance (highest standard) - Mean Absorbance (Zero standard)}} \times 100$$

Using semi-log graph, plot % bound on the vertical axis against HCP concentration on the horizontal axis for each of the calibrators and draw a smooth line curve through the points. HCP concentrations for the unknown may then be estimated from the line by interpolation.

Typical Data:
This data is for illustration only and must not be used for the calculation of any sample result.

Std. (ng/ml)	Abs1	Abs2	Mean Abs.	B/Bo %	Result (X with corrected dilution Factor (ng/ml)
0	0.418	0.440	0.429	100	
1	0.486	0.486	0.486	96.7	
4	0.567	0.560	0.564	92.2	
20	0.913	0.933	0.923	71.4	
75	1.440	1.464	1.452	40.8	
250	2.148	2.163	2.156		
Sample 1 (1:100)	0.543	0.514	0.529	94.20	290
Sample 2 (1:200)	0.479	0.514	0.497	96.10	380
Sample 3 (1:400)	0.457	0.475	0.466	97.80	440

For Example
Sample 1 = (2.156 – 0.529) / (2.156 – 0.429) x 100 = 94.20 %