



Title: Quantitation of CHO Host Cell Protein Using the Cygnus Inc ELISA Kit

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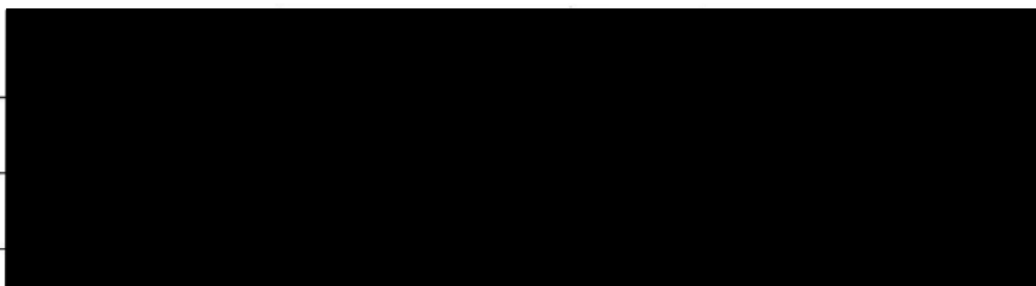


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1.0 Purpose

This SOP describes the use of the Cygnus, Inc., ELISA kit to determine the presence of Chinese Hamster Ovary (CHO) Host Cell Protein contamination in products manufactured by recombinant expression in CHO host cells.

2.0 Scope

This SOP applies to Process Analytics/Quality Control (PA/QC) personnel who will perform the CHO Host Cell Protein ELISA following this procedure.

3.0 Authority and Responsibility

- 3.1 The Director, Process Analytics/Quality Control (PA/QC) has the authority to define this procedure.

- 3.2 PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).
- 3.3 PA/QC personnel are responsible for performance of this procedure.
- 3.4 PA/QC is responsible for reviewing the data and documentation of the results of this procedure.
- 3.5 BQA is responsible for quality oversight of this procedure.

4.0 Supplies and Reagents

- 4.1 CHO Host Cell Protein 3rd Generation ELISA kit, (Biopharmaceutical Development Program) BDP PN 30965, Cygnus Technologies, Catalog Number F550.
- 4.2 Sample Diluent, BDP PN 30406, Cygnus Technologies, Catalog Number I028.
- 4.3 Single-channel and multi-channel pipettors.
- 4.4 Aerosol Barrier Pipet tips, 2-200 μ L, BDP PN 20673 and 1-1000 μ L, BDP PN 20769, VWR, or BDP approved equivalent.
- 4.5 Microtiter Plate Shaker, VWR Catalog Number 57019-600 or BDP approved equivalent.
- 4.6 Optical Adhesive Covers, BDP PN 21142 or BDP approved equivalent.
- 4.7 ZiplocTM Bag, BDP PN 20339, or BDP approved equivalent.
- 4.8 Reagent Reservoirs, BDP PN 20270, or BDP approved equivalent.
- 4.9 Microcentrifuge Tubes, 0.5 mL, BDP PN 21369, or BDP approved equivalent.
- 4.10 Ultrapure Water, or BDP approved equivalent.
- 4.11 Liter Graduated Cylinder for wash solution.
- 4.12 Squirt/Wash Bottle with the tip cut off.
- 4.13 Kaydry EX-L Kimwipes, BDP PN 21493, or BDP approved equivalent.
- 4.14 Labsystems iEMS Microtiter Plate Reader MF with Ascent software version 2.4.2, Model Number 1401, BDP MEF 66160, or BDP approved equivalent.

5.0 General Comments

- 5.1 Pipetting accuracy and reproducibility are critical for the success of this assay.
- 5.2 Use new pipette tips for each pipetting procedure (between dilutions).
- 5.3 Avoid contamination of the workspace when handling standards, ejecting pipette tips, etc. Pipette tips possessing an aerosol barrier must be used.
- 5.4 Good organization and attention to detail are essential to avoid confusion of sample identities and data.

6.0 Preparation

NOTE: The reagents are stored at 2 to 8°C. Bring all reagents to ambient temperature (up to one hour). All standards, controls, and samples will be assayed in duplicate. The total volume of the samples must take this into account.

- 6.1 Sample Preparation: Linear Range of Assay = 0 – 100 ng/mL. See Cygnus Kit Insert (**Attachment 5**).

- 6.1.1 Prepare the test article immediately before use undiluted and at recommended 1:5 and 1:50 dilutions using Sample Diluent. If the absorbance results indicate that the value for the 1:50 dilution is greater than 100 ng/mL, then repeat the ELISA assay with appropriately diluted samples. (The original data is attached to the QC Test Request.) An example for making dilutions is the following.
- For duplicate measurements of spiked and unspiked diluted test article (a total of four 50 μ L aliquots), add 60 μ L of test article to 240 μ L of Sample Diluent in a microcentrifuge tube.
 - A 1:50 dilution can be prepared by adding 30 μ L of the 1:5 diluted test article to 270 μ L of Sample Diluent.
- 6.1.2 Prepare the spiked sample of the test article. Spiked samples can be prepared by one of the following ways.
- A spiked sample can be prepared in microcentrifuge tubes prior to loading on the plate. For a spiked concentration of 25 ng/mL, perform a 4x dilution using the 100 ng/mL Cygnus, Inc. standard. For example, add 25 μ L of the 100 ng/mL standard to 75 μ L of the sample test article. Vortex well.
 - A spiked sample can also be prepared directly in the microtiter strip well. For a spiked concentration of 25 ng/mL, perform a 4x dilution in the well using the 100 ng/mL Cygnus, Inc. standard. For example, add 12.5 μ L of the 100 ng/mL standard directly into the well containing 37.5 μ L of the sample test article. Draw the sample and the spike up and down in the pipette tip 2 or 3 times for thorough mixing. Use a new pipette tip with each well.

6.2 Standard Preparation

- 6.2.1 Prepared standards at 0, 1, 3, 12, 40 and 100 ng/mL are included in the Cygnus CHO Host Cell Protein ELISA kit. To increase the number of points on the standard curve, one more standard, either a 6 ng/mL or a 20 ng/mL standard, is prepared fresh immediately before use.

For example:

- To prepare a 6 ng/mL standard, dilute the 12 ng/mL standard with Sample Diluent. Add 25 μ L of the 12 ng/mL standard to 25 μ L of the diluent directly in the well. This will need to be done in duplicate. Draw the liquid up and down in the pipette tip 2-3 times for thorough mixing. Use a new pipette tip with each well.
- A 20 ng/mL standard can be prepared as above, using the 40 ng/mL standard.

6.3 Positive, Negative, and Spike Control Preparation

- 6.3.1 The positive control will be either the 6 ng/mL, or 20 ng/mL standard that was prepared.
- 6.3.2 The negative control is the Sample Diluent.
- 6.3.3 A spiked control can be prepared in one of the following ways.
- A spiked control can be prepared in microcentrifuge tubes prior to loading on the plate. For a spiked concentration of 25 ng/mL, perform a 4x dilution using

the 100 ng/mL Cygnus, Inc. standard. For example, add 50 μ L of the 100 ng/mL standard to 150 μ L of Sample Diluent. Vortex well.

- A spiked control sample can also be prepared directly in the microtiter strip well. For a spiked concentration of 25 ng/mL, perform a 4x dilution using the 100 ng/mL Cygnus, Inc. standard. For example, add 12.5 μ L of the 100 ng/mL standard directly in the well containing 37.5 μ L of Sample Diluent. Draw the liquid up and down in the pipette tip 2-3 times for thorough mixing. Use a new pipette tip with each well.

6.4 Wash Solution Preparation

- 6.4.1 Empty the entire contents of the wash solution bottle (50 mL) into a 1 L graduated cylinder. Bring to 1 L with Ultrapure water. Log the wash solution in the PA/QC Solutions Logbook and label the solution bottle per **SOP 22702 - Solutions Used in Process Analytics**.

7.0 Procedure

This procedure is taken from the Cygnus Kit Insert (**Attachment 5**).

- 7.1 Prepare an ELISA Worklist (**Attachment 1**) by labeling the wells where the samples and standards will be placed. Count the microtiter strips needed. Remove the required number of microtiter strips from the kit and place them in the provided frame.
- 7.2 Pipette 100 μ L of the **anti-CHO:HRP** into each well using a multi-channel pipettor.
- 7.3 Pipette 50 μ L of **standards, controls and samples** into the wells indicated on the ELISA Worklist. All standards, controls and samples must be assayed in at least duplicate.
- 7.4 Carefully place the plate into a Ziploc™ bag.
- 7.5 Transfer to the microtiter plate shaker and incubate at room temperature for 2 hours \pm 2 minutes at a setting of 400-600 rpm.
- 7.6 Perform Manual Plate Wash. Flip contents from plate to waste and wash 4 times using a squirt bottle or by pipetting. Fill all wells to capacity with each wash. Blot the plate inverted on absorbent paper after each wash.
- 7.7 Pipette 100 μ L of **TMB Substrate**.
- NOTE:** If the substrate has a distinct blue color prior to the assay, there is a high probability that the substrate was contaminated. If this appears to be the case, read the absorbance of 100 μ L of substrate plus 100 μ L of Stop Solution against a water blank. If the absorbance is greater than 0.1, obtain a new substrate so the sensitivity of the assay will not be compromised.
- 7.8 Place the microtiter plate into a new Ziploc™ bag. Incubate at room temperature for 30 \pm 1 minutes. Do not shake the plate during this step.
- 7.9 Add 100 μ L of Stop Solution to each well. Pipet up and down 2-3 times to mix. Use fresh pipette tips with each new row or column of wells.
- 7.10 Read absorbance at 450/620 nm, blanking on the zero standard. Refer to **SOP 22100 - Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser**, for analysis of the data.

8.0 Data Analysis and Acceptable Criteria

After the Ascent CHO Host Cell Protein Results (**Attachment 2**) have been printed from the ELISA run, the results will need to be manually entered into an Excel Summary Worksheet (**Attachment 3**). The Summary Worksheet is also in a Microsoft Excel format, and the requested information is transcribed from the report generated by the plate reader and calculated by the Excel application. The acceptable spike recoveries and %CV's are calculated in this Summary Worksheet.

- 8.1 Open the CHO HCP Template ([REDACTED] Template)
- 8.2 Fill in the top portion of the summary sheet with all appropriate information, including QC number, Analyst, Date, Sample ID, Lot Number, Kit Lot Number, and Expiration Date.
- 8.3 Fill in the "CHO HCP Concentration" section with appropriate measured concentration for replicate 1 and 2 from the calculation column of the Ascent ELISA Results (**Attachment 2**). The "Corrected Concentration," "Average," and "% CV" will automatically be calculated on the spreadsheet. These numbers can be compared to those on the Ascent ELISA Results. They should be approximately the same depending upon rounding numbers. The "%CV" should be less than 25% for samples > 3 ng/mL.
- 8.4 Fill in the "Expected" and "Found" Positive Control and the "% CV" from the Ascent ELISA Results. The positive control is the standard (6 ng/mL or 20 ng/mL) that the analyst prepared and included in the standard curve. The "%CV" should be less than 25%. The "Found" positive control should be $100 \pm 30\%$ of what is "Expected."
- 8.5 Fill in the "Found" Negative Control from the Ascent ELISA Result. The negative control is the diluent used to make the sample dilutions. This number should be close to 0 or less than minimum (< min).
- 8.6 Fill in the "Found" Spike Control and the "% CV." The spike control is the diluent used to make the dilutions spiked with a known amount of standard. The "%CV" should be less than 25%. The "Found" spike control should be $100 \pm 30\%$ of what is "Expected."
- 8.7 Fill in replicate 1 and 2 for each dilution with the "Spiked Test Article Concentrations." These numbers are found on the Ascent ELISA Results under the "Calc." column for the spiked samples. These numbers are not corrected for dilutions. The "Average" and "% CV" will automatically be calculated and should be approximately the same as on the Ascent ELISA Results. The % CV should be less than 25%.
- 8.8 The test article must exhibit dilution-corrected analyte concentrations that vary no more than $\pm 50\%$ between dilutions. Avoid consideration of the dilutional data where the assay value before dilution correction falls below the lowest standard (1 ng/mL) for the CHO HCP assay. The first dilution-corrected concentration exhibiting dilutional linearity is reported. In the following example 8.2 ng/mL would be the reported result.

Example:

Undilute sample:	8.2 ng/mL	N/A
1:5 dilution:	6.5 ng/mL	79.3% of previous
1:50 dilution:	2.3 ng/mL	35.4% of previous

- 8.9 The "Percent Spike Recovery of Test Article" will be automatically calculated. The %CV should be less than 25% and the average recovery should be $100 \pm 30\%$.

- 8.10 If %CV and recoveries for the assay controls do not meet criteria, then the assay is invalid and will need to be rerun.
- 8.11 Save the Excel summary worksheet data, including the QC Test Request number in the file name.

9.0 Documentation

- 9.1 Print out the Excel Summary Worksheet (**Attachment 3**). Sign and date the worksheet and attach it to the QC Test Request Form with a copy of the Ascent CHO Host Cell Protein ELISA Results (**Attachment 2**).
- 9.2 The lowest dilution meeting the criteria listed above will be recorded on the QC Test Request Form.
NOTE: If the result is less than the lowest standard, it is recorded as < 1 ng/mL.
- 9.3 Record all solution preparation on **Form 23111-01, CHO Host Cell Protein ELISA Preparation Form (Attachment 5)**, and include with the QC Test Request Form.

10.0 References

- 10.1 Cygnus Technologies CHO Host Cell Protein ELISA Kit Instructions (**Attachment 5**).
- 10.2 Cygnus Suggested Manual Microtiter Plate Washing Procedure.
- 10.3 **SOP 22100** *Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser*
- 10.4 **SOP 22702** *Solutions Used in Process Analytics*

ATTACHMENT 1

Sample ELISA Worklist

H	G	F	E	D	C	B	A	↑
100 ng/mL Standard	40 ng/mL Standard	20 ng/mL Standard	12 ng/mL Standard	3 ng/mL Standard	1 ng/mL Standard	0 ng/mL Standard	BLANK 0ng/mL Std	1
100 ng/mL Standard	40 ng/mL Standard	20 ng/mL Standard	12 ng/mL Standard	3 ng/mL Standard	1 ng/mL Standard	0 ng/mL Standard	BLANK 0ng/mL Std	2
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED NEGATIVE CONTROL Sample diluent	NEGATIVE CONTROL Sample diluent	3
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED NEGATIVE CONTROL Sample diluent	NEGATIVE CONTROL Sample diluent	4
								5
								6
								7
								8
								9
								10
								11
								12

Assay:

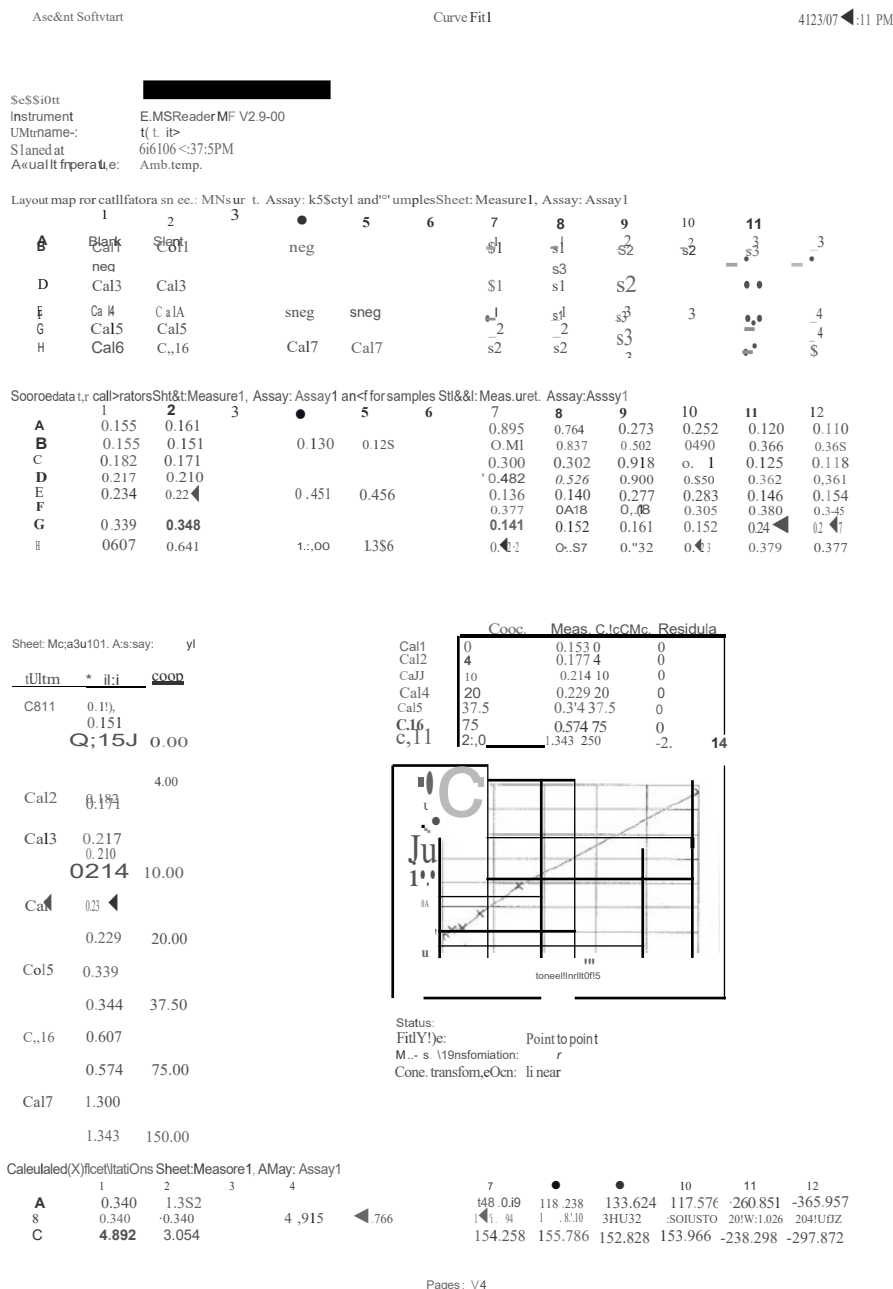
Sample ID:

Date:

Analyst:

ATTACHMENT 2

Sample Ascent CHO Host Cell Protein ELISA Results



ATTACHMENT 3 Sample EXCEL Summary Worksheet

Summary of CHO HCP ELISA Results

QC Number: QC060567 Analyst: Dale Ruby Date: 11/11/2018
 Sample ID: ID#1: Mab Eluate Lot #: L0000000
 Kit Lot#: 6412 Expiration Date: 5/31/2020

I. CHO HCP Concentration (ng/ml)						
Acceptable Criteria: %CV <25% for samples >4ng/mL						
Dilution Factor	Measured Concentration		Corrected Concentration		Average	%CV
	Replicate 1	Replicate 2	Replicate 1	Replicate 2		
1.00	7.86	8.54	7.86	8.54	8.20	5.86
5.00	1.19	1.41	5.95	7.05	6.50	11.97
50.00	0.04	0.05	2.15	2.45	2.30	68.75
Final Result:					8.20	ng/mL
*n/c = not calculated Corrected Concentration = Measured Concentration x dilution factor						

Calculated Concentration of "CHO HCP Spiking Solution" (ng/ml): 100.0

Correlation Coefficient of Standard Curve: point to point

Acceptable Criteria: %Recovery (100% ± 30) and %CV <25%

	Expected	Found	%Recovery	%CV
Avg. Positive Control (ng/ml):	20.00	20.05	100.25%	7.31
Avg. Negative Control (ng/ml): (dilution buffer only)	0.00	<min	N/A	16.32
Avg. Spike Control (ng/ml): (spiked dilution buffer)	25.00	23.24	92.96%	5.64
[[0.0125ml x (conc. of "CHO HCP Spiking Sol'n." (in ng/ml))] / 0.05 ml] x 100%				

Dilution Linearity

Dilution Factor	Average Dilution Corrected Concentration	Percent Change in Dilution Corrected Concentration from Previous Value
1.00	8.20	N/A
5.00	6.50	79.3
50.00	2.30	35.4

Acceptability Criteria: Dilution Corrected Concentration 50 - 150% of Previous Value

Spiked Test Article Concentrations (ng/ml; not corrected for dilution):

Acceptable Criteria: %CV <25%					
Dilution	Replicate 1	Replicate 2	Average	%CV	
1	30.99	31.55	31.27	1.27	
5	24.72	26.15	25.44	3.98	
50	26.45	25.32	25.89	3.09	

Percent Spike Recovery of Test Article:

Acceptable Criteria: Average Recovery (100% ± 30) and %CV <25%					
Dilution	Replicate 1	Replicate 2	Average	%CV	
1	100.31	100.46	100.38	0.11	
5	98.88	104.60	101.74	3.98	
50	102.15	97.17	99.66	3.54	

Calculated by: Spiked test article concentration, ng/ml + [(0.0375 ml) * (Test article measured concentration, ng/ml) + (0.0125 ml) * (Concentration of "CHO HCP Spiking Solution", ng/ml)] / 0.05 ml] x 100%

Analyst/Date: _____

Reviewed By/Date: _____

ATTACHMENT 4
Form 23111-01, CHO Host Cell Protein ELISA Preparation

NCI-Frederick
Form No.: 23111-01
SOP No.: 23111
Revision 01: APR 16 2018

Quantitation of CHO Host Cell Protein ELISA PREPARATION FORM

Page 1 of 2

QC Number: _____ Operator: _____ Date: _____ Kit catalog#: _____

Plate Reader MEF Number: _____ Calibration Due Date: _____

Diluent (I028): Lot# _____ Exp. Date: _____**Test sample Dilutions:****Test Sample #1**

Name: _____
Lot # _____
Protein Concentration: _____ mg/mL

Initial dilution: _____
Volume of diluent: _____ μ L
Volume of test sample: _____ μ L

Second dilution: _____
Volume of diluent: _____ μ L
Volume of initial diluted test sample _____ μ L

Third Dilution _____
Volume of Diluent: _____ μ L
Volume of Second Diluent sample _____ μ L

Test Sample #3

Name: _____
Lot # _____
Protein Concentration: _____ mg/mL

Initial dilution: _____
Volume of diluent: _____ μ L
Volume of test sample: _____ μ L

Second dilution: _____
Volume of diluent: _____ μ L
Volume of initial diluted test sample _____ μ L

Third Dilution _____
Volume of Diluent: _____ μ L
Volume of Second Diluent sample _____ μ L

Test Sample #2

Name: _____
Lot # _____
Protein Concentration: _____ mg/mL

Initial dilution: _____
Volume of diluent: _____ μ L
Volume of test sample: _____ μ L

Second dilution: _____
Volume of diluent: _____ μ L
Volume of initial diluted test sample _____ μ L

Third Dilution _____
Volume of Diluent: _____ μ L
Volume of Second Diluent sample _____ μ L

Test Sample #4

Name: _____
Lot # _____
Protein Concentration: _____ mg/mL

Initial dilution: _____
Volume of diluent: _____ μ L
Volume of test sample: _____ μ L

Second dilution: _____
Volume of diluent: _____ μ L
Volume of initial diluted test sample _____ μ L

Third Dilution _____
Volume of Diluent: _____ μ L
Volume of Second Diluent sample _____ μ L

ATTACHMENT 4 (Continued)

Form 23111-01, CHO Host Cell Protein ELISA Preparation

NCI-Frederick
Form No.: 23111-01
SOP No.: 23111
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Quantitation of CHO Host Cell Protein ELISA PREPARATION FORM

Page 2 of 2

Positive Control (Prepared Standard)

Expiration Date: _____
Concentration: _____ ng/mL
Std: _____ μ L
Sample Dilution Buffer: _____ μ L

Negative Control Spike

Expiration Date: _____
Concentration: _____ ng/mL
Std: _____ μ L
Sample Dilution Buffer: _____ μ L

anti-CHO:HRP Antibody (#F551)

Lot# _____
Exp. Date: _____
Volume of antibody added to wells: _____ μ L
Incubation Time: _____

Microtiter plate shaker: Make: _____ Model: _____

Washing: Number of Washes _____ Volume added to wells (300-400 μ L) _____ μ L

TMB Substrate (#F005)

Lot # _____
Expiration Date: _____
Volume of substrate added to wells: _____
Incubation Time: _____

Stop Solution (#F006)

Lot # _____
Expiration Date: _____
Volume of Stop Solution added to wells: _____

Item	MEF/BDP PN	BDP Release Number	Expiration Date
Pipettes			
Pipette Tips			
Microcentrifuge Tubes			
Optical Adhesive Covers			
Plastic (Ziploc) Bags			
Reagent reservoirs			

Data Storage

File Name: _____

Performed by/Date: _____ Reviewed by/Date: _____

ATTACHMENT 5

Cygnus Technologies Inc. CHO ELISA Kit Insert



CHO Host Cell Proteins 3rd Generation

Immunoenzymetric Assay for the Measurement of CHO Host Cell Proteins Catalog # F550

Intended Use

This kit is intended for use in determining the presence of host cell protein contamination in products manufactured by expression in the CHO cell line. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals. This is the 3rd Generation ELISA kit for CHO HCP detection. Cygnus continues to market two earlier generation kits, Cat #s F015 and CM015 for CHO HCP detection. While those kits are broadly reactive to CHO HCPs among all strains and have been successfully validated for a range of drug substances, this 3rd Generation assay uses an even more broadly reactive antibody. The 3rd Generation Cat# F550 kit offers greater sensitivity and a more linear standard curve while incorporating a number of improvements to overcome matrix interference and sample dilutional linearity problems sometimes encountered in the two older kits.

Summary and Explanation

Expression of therapeutic proteins in CHO cells is a cost effective method for production of commercial quantities of a drug substance. The manufacturing and purification process of these products leaves the potential for contamination by host cell proteins (HCPs) from CHO cells. Such contamination can reduce the efficacy of the therapeutic agent and result in adverse toxic or immunological reactions and thus it is desirable to reduce HCP contamination to the lowest levels practical.

Immunological methods using antibodies to HCPs such as Western Blot and ELISA are conventionally accepted. While Western blot is a useful method aiding in the identity of HCPs, it suffers from a number of limitations. Western blot is a complex and technique dependent procedure requiring subjective interpretation of results. Furthermore, it is essentially a qualitative method and does not lend itself to obtaining quantitative answers. The sensitivity of Western blot is severely limited by the volume of sample that can be tested, and by interference from the presence of high concentrations of the intended product. While Western Blot may be able to detect HCPs in samples from upstream in the purification process, it often lacks adequate sensitivity and specificity to detect HCPs in purified downstream

and final product. The microtiter plate immunoenzymetric assay (ELISA) method employed in this kit overcomes the limitations of Western blots providing on the order of 100 fold better sensitivity. This simple to use, objective, and semi-quantitative ELISA is a powerful method to aid in optimal purification process development, process control, routine quality control, and product release testing. This kit is "generic" in the sense that it is intended to react with essentially all of the HCPs that could contaminate the product independent of the purification process. The antibodies have been generated in goats and affinity purified using CHO HCPs found in protein free conditioned media. Western blot, both 1 & 2 dimensional, was used as a preliminary method and established that the antibodies reacted to the majority of HCP bands resolved by the PAGE separation. Further characterization to more highly resolved proteins was accomplished by a method we term 2D HPLC-ELISA. We find this method far superior to 2D Western blot in sensitivity and specificity. The 2D HPLC fractionation of conditioned media derived HCPs from 2 different CHO strains (CHO-S & K1) showed reactivity to ~750 fractions presumed to be individual HCPs. For more information on this 2D HPLC-ELISA analysis please contact our Technical Services department.

Special procedures were utilized in the generation of these antibodies to insure that low molecular weight and less immunogenic contaminants as well as high molecular weight components would be represented. As such, this kit can be used as a process development tool to monitor the optimal removal of host cell contaminants as well as in routine final product release.

This highly sensitive ELISA kit has been qualified for testing of final product HCPs using actual in-process and final drug substance samples from 5 different drug products including monoclonal antibodies and other therapeutic proteins. The assay had sensitivity to detect HCP in all 5 final drug substances showing acceptable dilutional linearity and spike recovery. Based on this experience this assay should have application as a multi-use assay for other products expressed in CHO cells. Each user of this kit is encouraged to perform a similar validation study to demonstrate it meets their analytical needs. Provided this kit can be satisfactorily validated for your samples, the application of a more process specific assay may not be necessary in that such an assay would only provide information redundant

ATTACHMENT 5 (Continued)

Cygnus CHO HCP ELISA Kit Insert

to this generic assay. However, if your validation studies indicate the antibodies in this kit are not sufficiently reactive with your process specific HCPs it may be desirable to also develop a more process specific ELISA. This later generation assay may require the use of a more specific and defined antisera. Alternatively, if the polyclonal antibody used in this kit provides sufficient sensitivity and broad antigen reactivity, it may be possible to substitute the standards used in this kit for ones made from the contaminants that typically co-purify through your purification process and thus achieve better accuracy for process specific HCPs. The use of a process specific assay with more defined antigens and antibodies in theory may yield better specificity, however such an assay runs the risk of being too specific in that it may fail to detect new or atypical contaminants that might result from some process irregularity or change. For this reason it is recommended that a broadly reactive "generic" host cell protein assay be used as part of the final product purity analysis even when a process specific assay is available. If you deem a more process specific assay is necessary, Cygnus Technologies is available to apply its proven technologies to develop such antibodies and assays on custom basis.

Principle of the Procedure

The CHO assay is a two-site immunoenzymetric assay. Samples containing CHO HCPs are reacted simultaneously with a horseradish peroxidase (HRP) enzyme labeled anti-CHO antibody (goat polyclonal) in microtiter strips coated with an affinity purified capture anti-CHO antibody. The immunological reactions result in the formation of a sandwich complex of solid phase antibody-HCP-enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactants. The substrate, tetramethylbenzidine (TMB) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of CHO HCPs present.

Reagents & Materials Provided

Component	Product #
Anti-CHO:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F551
Anti-CHO coated microtiter strips 12x8 well strips in a bag with desiccant	F552*
CHO HCP Standards CHO HCPs in bovine serum albumin with preservative. Standards at 0, 1, 3, 12, 40, and 100ng/mL. 1 mL/vial	F553
Stop Solution 0.5M sulfuric acid. 1x12mL	F006
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	F004

*All components can be purchased separately except # F552.

800-F550, Rev. 3, 21JUL2015

CHO HCP 3G ELISA Product Insert

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Materials & Equipment Required But Not Provided

Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)*

Pipettors - 50µL and 100µL

Repeating or multichannel pipettor - 100µL

Microtiter plate rotator (400-600 rpm)

Sample Diluent (recommended Cat # I028)

Distilled water

1 liter wash bottle for diluted wash solution

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed.
- The substrate reagent should not be used if its stopped absorbance at 450nm is greater than 0.1.
- Reconstituted wash solution is stable until the expiration date of the kit.
- After prolonged storage, you may notice a salt precipitate and/or yellowing of the wash concentrate. These changes will not impact assay performance. To dissolve the precipitate, mix the wash concentrate thoroughly and dilute as directed in the 'Preparation of Reagents' section. Reconstituted wash solution is stable until the expiration date of the kit.

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H₂SO₄. Avoid contact with eyes, skin, and clothing. At the concentrations used in this kit, none of the other reagents are believed to be harmful.
- This kit should only be used by qualified technicians.

Preparation of Reagents

- Bring all reagents to room temperature.
- Dilute wash concentrate to 1 liter in distilled water, label with kit lot and expiration date, and store at 4°C.

Procedural Notes

- Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. We advise against the use of automated or other manually operated vacuum aspiration devices for washing plates as these may result in lower specific absorbances, higher non-specific absorbance, and more variable precision. The manual

ATTACHMENT 5 (Continued)

Cygnus CHO HCP ELISA Kit Insert

wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision. If duplicate CVs are poor, or if the absorbance of the '0' standard is greater than 0.300, evaluate plate washing procedure for proper performance.

2. High Dose Hook Effect or poor dilutional linearity may be observed in samples with very high concentrations of HCP. High Dose Hook Effect is due to insufficient excess of antibody for very high concentrations of HCPs present in samples upstream in the purification process. Samples greater than 1mg/mL may give absorbances less than the 100ng/mL standard. It is also possible for samples to have certain HCPs in concentrations exceeding the amount of antibody for that particular HCP. In such cases the absorbance of the sample at all dilutions may be lower than the highest standard in the kit, however these samples will fail to show acceptable dilutional linearity/parallelism as evidenced by an apparent increase in dilution corrected HCP concentration with increasing dilution. High Dose Hook and poor dilutional linearity are most likely to be encountered from samples early in the purification process. If a hook effect is possible, samples should also be assayed diluted. If the HCP concentration of the undiluted sample is less than the diluted sample this may be indicative of the hook effect. Such samples should be diluted at least to the minimum required dilutions (MRDs) as established by your validation studies using your actual final and in-process drug samples. The MRD is the first dilution at which all subsequent dilutions yield the same HCP value within the statistical limits of assay precision. The HCP value to be reported for such samples is the dilution corrected value at or greater than the established MRD. The diluent used should be compatible with accurate recovery. The preferred diluent is our Cat# I028 available in 100mL, 500mL, or 1 liter bottles. This is the same material used to prepare the kit standards. As the sample is diluted in I028, its matrix begins to approach that of the standards, thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for non-specific binding and recovery by using them to dilute the 100ng/mL standard, as described in the "Limitations" section below.

Limitations

- Before relying exclusively on this assay to detect host cell proteins, each laboratory should validate that the kit antibodies and assay procedure yield acceptable specificity, accuracy, and precision. A suggested protocol for this validation can be obtained from our Technical Services Department or our web site.
- The standards used in this assay are comprised of CHO HCPs obtained after the culture of null CHO cells in protein free culture media. 1D Western blot

analysis of the antibodies used in this kit demonstrates that they recognize the majority of distinct PAGE separated bands seen using sensitive protein staining methods like silver stain or colloidal gold. 2D HPLC fractionation of this same conditioned media showed >750 protein fractions reactive in this ELISA. Because the majority of HCPs will show antigenic conservation among all strains of CHO this kit should be adequately reactive to the vast majority of HCPs from your transfected cell line. However, there can be no guarantee that this assay will detect all proteins or protein fragments from your process. If you desire a much more sensitive and specific method than western blot to detect the reactivity of the antibodies in this kit to your individual HCPs Cygnus is pleased to offer a service for fractionation of HCPs using 2-D HPLC methods followed by detection in ELISA.

- Certain sample matrices may interfere in this assay. The standards used in this kit attempt to simulate typical sample protein and matrices. However the potential exists that the product itself or other components in the sample matrix may result in either positive or negative interference in this assay. High or low pH, detergents, urea, high salt concentrations, and organic solvents are some of the known interference factors. It is advised to test all sample matrices for interference by diluting the 100ng/mL standard, 1 part to 4 parts of the matrix containing no or very low HCP contaminants. This diluted standard when assayed as an unknown, should give an added HCP value in the range of 15 to 25 ng/mL. Consult Cygnus Technologies Technical Service Department for advice on how to quantitate the assay in problematic matrices.
- Avoid the assay of samples containing sodium azide (NaN₃) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

Assay Protocol

- The HRP conjugate will have a cloudy appearance. This is normal and does not indicate contamination. Overtime, you may observe a slight precipitate. This precipitate is inconsequential to assay results. We suggest a simple inversion of the bottle to re-suspend it.
- The assay is very robust such that assay variables like incubation times, sample size, and other sequential incubation schemes can be altered to manipulate assay performance for more sensitivity, increased upper analytical range, or reduced sample matrix interference. Before modifying the protocol from what is recommended, you are advised to contact

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Technical Services for input on the best way to achieve your desired goals.

- The protocol specifies use of an approved microtiter plate shaker or rotator for the immunological steps. These can be purchased from most laboratory supply companies. If you do not have such a device, it is possible to incubate the plate without shaking however it will be necessary to extend the immunological incubation step in the plate by about one hour in order to achieve comparable results to the shaking protocol. **Do not shake during the 30 minute substrate incubation step, as this may result in higher backgrounds and worse precision.**
- Bring all reagents to room temperature. Set-up plate spectrophotometer to read dual wavelength at 450nm for the test wavelength and ~650nm for the reference.
- Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site. A video demonstration of proper plate washing technique is also available in the 'Technical Help' section of our web site.
- All standards, controls, and samples should be assayed at least in duplicate.
- Maintain a repetitive timing sequence from well to well for all assay steps to insure that all incubation times are the same for each well.
- Make a work list for each assay to identify the location of each standard, control, and sample.
- It is recommended that your laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct. **You are strongly urged to make controls in your typical sample matrix using HCPs derived from your cell line. These controls can be aliquoted into single use vials and stored frozen for long-term stability.**
- If the substrate has a distinct blue color prior to assay it may have been contaminated. If the absorbance of 100µL of substrate plus 100µL of stop against a water blank is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.
- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

- Pipette 100µL of anti-CHO:HRP (#F551) into each well.
- Pipette 50µL of standards, controls and samples into wells indicated on work list.
- Cover & incubate on rotator at 400-600rpm for 2 hours at room temperature, 24°C ± 4°C.
- Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate or use of vacuum aspiration devices in an attempt to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle or by pipetting in ~350µL. Dump and tap again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding substrate.
- Pipette 100µL of TMB substrate (#F005).
- Incubate at room temperature for 30 minutes. **DO NOT SHAKE.**
- Pipette 100µL of Stop Solution (#F006).
- Read absorbance at 450/650nm.

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL HCP equivs.
1A	Zero Std	0.122	0.125	
1B	Zero Std	0.128		
1C	1ng/mL	0.167	0.169	
1D	1ng/mL	0.171		
1E	3ng/mL	0.253	0.250	
1F	3ng/mL	0.246		
1G	12ng/mL	0.571	0.575	
1H	12ng/mL	0.579		
2A	40ng/mL	1.450	1.436	
2B	40ng/mL	1.422		
2C	100ng/mL	2.709	2.722	
2D	100ng/mL	2.734		
2E	sample A	0.248	0.240	2.76
2F	sample A	0.232		
2G	sample B	1.906	1.961	64.5
2H	sample B	2.016		

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL "total immuno-reactive HCP equivalents". This data reduction may be performed through computer methods using curve-fitting

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routines such as point-to-point, cubic spline, or 4 parameter logistic fit. **Do not use linear regression analysis to interpolate values for samples as this may lead to significant inaccuracies!** Data may also be manually reduced by plotting the absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line. Absorbances of samples are then interpolated from this standard curve.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 3-100ng/mL. CVs for samples <3 ng/mL may be greater than 10%.
- For optimal performance the absorbance of the substrate when blanked against water should be < 0.1.
- It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. A copy of this validation report can be obtained on our web site or by request. This validation is generic in nature and is intended to supplement but not replace certain user and product specific qualification and validation that should be performed by each laboratory. At a minimum each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your samples types containing process derived HCPs within or above the analytical range of this assay should be evaluated for dilutional linearity to insure that the assay is accurate and has sufficient antibody excess for your particular HCPs. Each laboratory and technician should also demonstrate competency in the assay by performing a precision study similar to that described below. A more detailed discussion of recommended user validation protocols can be obtained by contacting our Technical Services Department or at our web site.

Sensitivity

The lower limit of detection (LOD) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. LOD is ~0.3 ng/mL.

The lower limit of quantitation (LOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are <20%. The LOQ is ~1 ng/mL.

Specificity/Cross-Reactivity

Western blot and ELISA analysis against 2 commercial CHO cell lines identified as CHO-S and K1 indicate that most of the proteins are conserved among all lines. Therefore this assay should be useful for detecting HCPs from other CHO strains. Western blot, both 1 & 2 dimensional, is highly orthogonal to ELISA and to non-specific protein staining methods such as silver stain or colloidal gold. As such, 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. If you desire a much more sensitive and specific method than Western blot to detect the reactivity of the antibodies in this kit to your individual HCPs Cygnus is pleased to offer a service and/or consultation on fractionation of HCPs using 2 Dimensional HPLC methods followed by detection in the ELISA. This method has been shown to be much at least 100 fold more sensitive than Western blots in detecting antibody reactivity to individual HCPs. The same antibody as is used for both capture and HRP label can be purchased separately.

Cross reactivity to non-HCP components has not been extensively investigated with this kit. You should evaluate components in your samples for positive interferences such as cross reactivity and non-specific binding. Negative interference studies are described below.

Precision

Both intra (n=20 replicates) and inter-assay (n=10 assays) precision were determined on 3 pools with low (~3ng/mL), medium (~12ng/mL), and high concentrations (~60ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	6.9%	7.2%
Medium	2.7%	4.4%
High	3.7%	5.0%

Recovery/Interference Studies

Various buffer matrices commonly used in purification of therapeutic proteins and monoclonal antibodies as well as in-process and final formulation drug substances were evaluated by adding known amounts of CHO HCP preparation used to make the standards in this kit. All of these samples yielded acceptable recovery defined as between 80-120%. The standards used in this kit contain 8mg/mL of bovine serum albumin intended to simulate non-specific protein effects of most sample proteins. However, very high concentrations of some products may interfere in the accurate measurement of HCPs. In general, extremes in pH (<5.0 and >8.5), high

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salt concentration, high polysaccharide concentrations, urea, organic solvents, and most detergents can cause under-recovery. Each user should validate that their sample matrices yield accurate recovery. Such an experiment can be performed, by diluting the 100ng/mL standard provided with this kit, into the sample matrix in question as described in the "Limitations" section. Cygnus offers a more concentrated form of the HCP used to prepare the kits standards for your spike recovery and preparation of analyte controls.

Hook Capacity

Increasing concentrations of HCPs >100 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration yielding an absorbance reading less than the 100 ng/mL standard was ~1 mg/mL.

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the extraction and detection of CHO Host Cell DNA. The following kits are available:

- Residual Host Cell DNA extraction:
Cat # D100W, DNA Extraction Kit in 96 deep well plate
Cat # D100T, DNA Extraction Kit in microfuge tubes
- Extraction and PCR amplification of CHO Host Cell DNA for use with user supplied master mix:
Cat # D555W, DNA Extraction Kit in 96 deep well plate
Cat # D555T, DNA Extraction Kit in microfuge tubes
- Residual CHO Host Cell DNA extraction and detection using PicoGreen® dye:
Cat # D550W, DNA Extraction Kit in 96 deep well plate
Cat # D550T, DNA Extraction Kit in microfuge tubes

To place an order or to obtain additional product information contact *Cygnus Technologies*:

www.cygnustechnologies.com

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