Quantitation of HEK 293 Host Cell Protein Using the Cygnus Inc. ELISA Kit

SOP 23103 **Rev. 05**

Table of Contents

1.0 Purpose

This SOP describes the use of the Cygnus, Inc., ELISA kit to determine the presence of host cell protein contamination in products manufactured by recombinant expression in Human Embryonic Kidney cell line 293 (HEK 293) host cells.

2.0 Scope

This SOP applies to Process Analytics/Quality Control (PA/QC) personnel who will perform the HEK 293 Host Cell Protein ELISA following this procedure.

NOTE: It is required that new (not previously analyzed) test articles (Final product, bulk or in-process sample) are submitted for R&D testing to determine assay suitability for a particular test article. Conditions necessary for acceptable spike recovery and dilutional linearity must be established prior to GMP testing. It is also required that a sample of the **buffer** that the test article is in be submitted for testing as a blank along with the sample.

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 Materials and Equipment

4.1 HEK 293 Host Cell Protein ELISA kit, Biopharmaceutical Development Program (BDP) PN 30649, Cygnus Technologies, Catalog Number F650R.

4.2 Sample Diluent, BDP PN 30406, Cygnus Technologies, Catalog Number I028.

4.3 Calibrated multi-channel pipettor 30-300 µL.

4.4 Calibrated pipettors 2-200 µL and 100-1000 µL.

4.5 Aerosol Barrier Pipet tips, 2-200 µL, BDP PN 20673 and 1-1000 µL, BDP PN 20769, or BDP approved equivalent.

4.6 Microtiter plate shaker, VWR Catalog Number 57019-600, or BDP approved equivalent.

4.7 Ziploc™ 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 1 Liter Graduated Cylinder to prepare 1x wash solution

4.12 Squirt/wash bottle with the tip cut off.

4.13 Low-lint or lint-free absorbent paper, BDP PN 21493 or BDP approved equivalent.

4.14 Hand Held Aspirator, Corning Costar.

4.15 Clorox Bleach, BDP PN 20295.

4.16 Optical Adhesive Covers, BDP PN21142.

4.17 Blue Diapers, BDP PN 20382.

4.18 Biohazard Pipette Keeper, BDP PN 21338.

4.19 70% Isopropyl Alcohol, BDP PN 30129.

4.20 Dispatch, BDP PN 10167 and Cavicide, BDP PN 10167.

4.21 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 a new pipette tip for each pipetting procedure (between dilutions).

5.3 Avoid contamination of the workspace when handling HEK 293 Host Cell Protein Standards, ejecting pipettes, 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.

5.5 Certain sample matrices may interfere in this assay. Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration or extremes of pH (<5.0 and >8.5) may give erroneous results. A buffer blank is required to be submitted with the sample.

6.0 Preparation

The ELISA kit, wash buffer and diluent are stored at 2-8°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.

NOTE: If the sample being tested is marked biohazard on the QC test request, all steps need to be performed in a biological safety cabinet. Refer to ***SOP 17109 - Procedures for Safe Handling, Decontamination, and Spill Cleanup of Infectious or Potentially Infectious Materials.***

6.1 Sample Preparation: Linear Range of Assay= 0 – 200 ng/mL

6.1.1 Prepare the test article immediately before use, undiluted and at recommended dilutions of 1:5 and 1:50 using the sample diluent (see 4.2). If the absorbance results indicate that the HCP content for the 1:50 dilution is greater than 200 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:

6.1.1.1 For duplicate measurements of spiked and unspiked diluted test article (a total of four 50 µL aliquots), add 100 µL test article to 400 µL diluent in a microcentrifuge tube to give a 1:5 dilution.

6.1.1.2 A 1:50 dilution can be made up in microcentrifuge tubes by adding 50 µL of the 1:5 diluted test article to 450 µL diluent.

6.1.2 Prepare the spiked test article and a spiked-buffer control. These can be prepared as follows.

6.1.2.1 A spiked test article can be prepared in microcentrifuge tubes prior to loading on the plate. For a spiked concentration of 50 ng/mL HEK 293 HCP, do a 4x dilution using the 200 ng/mL Cygnus, Inc. standard. For example, add 50 µL of the 200 ng/mL standard to 150 µL of the sample test article prepared in step 4.1.1. Mix thoroughly. Do not vortex infectious samples.

A spiked-buffer control sample can be prepared in microcentrifuge tubes prior to loading on the plate. For a spiked concentration of 50 ng/mL HEK 293 HCP, do a 4x dilution using the 200 ng/mL Cygnus, Inc. standard. For example, add 50 µL of the 200 ng/mL standard to 150 µL of the bufferprovided by the requestor. This produces a 50 ng/mL concentration of HEK293 HCP in the spiked-buffer control sample. Mix thoroughly. Do not vortex infectious samples.

6.2 Standard Preparation

6.2.1 Prepared HCP standards at 0, 4, 10, 25, 75 and 200 ng/mL are included in the Cygnus HEK 293 HCP ELISA kit. To increase the number of points on the standard curve, one more standard, either a 12.5 ng/mL or a 37.5 ng/mL standard, is prepared fresh immediately before use.

6.2.2 For example:

6.2.2.1 To make a 12.5 ng/mL standard do a 1:1 dilution with the 25 ng/mL standard. Add 25 µL of the 25 ng/mL standard to 25 µL of the diluent directly into the well. This will need to be done in duplicate. Draw the sample and the diluent up and down in the pipette tip 2 or 3 times for thorough mixing. Use a new pipette tip with each well.

6.2.2.2 To make a 37.5 ng/mL standard do a 1:1 dilution with the 75 ng/mL standard. Add 25 µL of the 75 ng/mL standard to 25 µL of the diluent directly into the well. This will need to be done in duplicate. Draw the sample and the diluent up and down in the pipette tip 2 or 3 times for thorough mixing. Use a new pipette tip with each well.

6.3 Positive Control Preparation

6.3.1 The positive control will be either the 12.5 ng/mL or 37.5 ng/mL standard that was prepared in section 6.2.1.

6.4 Wash Solution Preparation

6.4.1 Empty the entire contents of the wash solution bottle into a 1 L graduated cylinder. Bring to 1 L with Ultrapure water. Log the wash solution in the BQC Solutions logbook. Label the solution bottle with the QCB number, initials, date prepared, and expiration date which are found on the label of the wash solution bottle. Refer to ***SOP 22702 - Solutions Used in Process Analytics***.

7.0 Procedure

The procedure is taken from the Cygnus Kit Insert (Attachment 4).

7.1 Prepare an ELISA Work list (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 µL of **anti-HEK 293:HRP** into each well.

7.2.1 Pour contents of **anti-HEK 293:HRP** bottle into a new reagent reservoir (4.8).

7.2.2 Use a multi-channel pipettor (30 – 300µL) to fill rows of wells. Use fresh pipette tips for each row.

7.3 Pipette 50 µL of the **standards, controls, and samples** into wells as indicated on the ELISA Work list (Attachment 1). All standards, controls and samples must be assayed in at least duplicate.

7.4 Carefully place the plate into a Ziploc bag (4.7).

7.5 Transfer to the microtiter plate shaker (4.6) and incubate for at least 2 hours at a setting between "4 to 6" (400-600 rpm).

NOTE: For safety reasons if the sample is a live virus, the plate will need to be washed with a hand-held aspirator. See step 7.7 for detailed instructions on handling and aspirating of the plate. For all other samples a manual plate procedure can be used. See step 7.6 below.

7.6 Using a manual microtiter plate procedure, wash the plate with at least 350 µL of diluted wash solution from step 6.4 and remove. Wash a total of 4 times as follows:

7.6.1 Remove the liquid from the plate as follows: Grab the plate from the bottom with the thumb in the middle of one side and the fingers on the other side. If the thumb and fingers slightly overlap the tabs on the ends of the middle strip or strips, the operator will usually be able to avoid having any strips fall out of the strip holder. Holding the plate over the sink, turn the plate upside down just as the operator rapidly accelerates the arm and hand downward. Abruptly stop the arm movement, causing the liquid to be forced from the strips into the sink. Repeat the removal motion a second time.

7.6.2 Blot and strike the plate. Immediately blot the upside-down plate onto the low-lint absorbent paper (4.13). Move the plate to an unused section of the blotting paper and allow it to drain upside down for approximately 30 seconds. Strike the plate hard 4 times over the unused areas of the paper. Do not be afraid to strike vigorously. Anything short of breaking the stripholder or strips is not too hard. Wash the plate. Use the squirt bottle (4.12) with the narrow portion of the tip cut off to give the largest possible orifice so that the flow will be generous and gentle. Fill all wells with the diluted wash solution provided with the kit (See 6.4). Remove the wash solution and blot and strike the plate as described in step 7.6.1 and 7.6.2. Repeat the washing, blotting, and striking procedure 3 more times for a total of 4 washes. After the last wash, let the plate rest upside down for at least 60 seconds to drain. Strike the plate again 4-6 times rotating the plate 180° between each strike. This rotation ensures that the ends of the plate receive on average the same energy and impact.

7.6.3 Wipe the bottom outside of all wells with clean absorbent paper to remove any liquid from the washing. If the washing technique has been performed correctly the center of each well should have a small film of liquid (< 1 microliter). If the film is not uniform in terms of the area between the wells, or a significant amount of liquid remains in the circular edge of the wells, then strike the plate more forcefully. Wells are now ready to have the substrate added to them. See step 7.8.

Do not add substrate near the sink location where the removal and striking has taken place since the washing procedure can generate aerosols that could re-contaminate the wells or substrate.

7.7 When the sample being tested is a **live virus** the wash protocol will need to be performed using a hand-held aspirator (See 4.14) with an 8-channel adapter for the first rinse step. The aspirator is designed for use with an in-house vacuum system and a collection vessel.

7.7.1 Connect the hose barb end of the aspirator hand piece to rubber tubing and connect the tubing to a collection bottle. The tubing to the vacuum source then connects to the collection bottle. When working with biohazardous samples, the collection bottle needs a safety trap or approved filter between it and the vacuum source.

7.7.2 Connect the 8-channel adapter to the hand piece of the aspirator. Use fresh disposable tips with each wash and removal step. Place the tips in the wells of the plate. The pressure sensitive red control button on the top of the hand piece controls the vacuum pressure through the aspirator. Press the red control button to aspirate the liquid from the wells. Dispose of tips in a biohazard pipette keeper (4.18).

7.7.3 Pour wash solution from step 6.4 into a reagent reservoir. Using a multi-channel pipettor with fresh disposable tips, pipette 300 µL into each row of wells in the plate. Dispose of tips in a biohazard pipette keeper.

7.7.4 Using the 8-channel aspirator with fresh disposable tips, aspirate the wash solution from each row of wells on the plate. This is the last time the aspirator will be used. Because the remaining virus samples were aspirated from the wells, the plate can now be washed and blotted manually.

7.7.5 Blot and strike the plate. Place a blue diaper (4.17) in the biological safety cabinet with a layer of low-lint absorbent paper (4.13) on top of it. Blot the upside-down plate onto the low-lint absorbent paper. This step is performed with your hand and plate underneath a second blue diaper. This is to eliminate any splashing that may occur while blotting and striking. After the plate is blotted upside down on the low-lint absorbent paper, move the plate to an unused section of the blotting paper and allow it to drain upside down for at least 30 seconds. Strike the plate hard four times over the unused areas of the paper. Do not be afraid to strike vigorously.

7.7.6 The plate is now ready to be washed again. Repeat steps 7.7.3 and 7.7.5 two more times for a total of three times. Be sure to use a fresh set of blue diapers and low-lint absorbent papers with each wash and blot step. After a total of four washes, one using the aspirator and three using the blotting and striking procedure, the plate is now ready for substrate to be added.

7.7.7 Cleaning and Decontamination. To clean the 8-channel aspirator, pour bleach into a reagent reservoir and aspirate. To rinse the bleach out, pour Direct-Q water into a reagent reservoir and aspirate. Dispose of all blue diapers and low-lint absorbent papers in a biohazard bag.

7.8 Pipette 100 µL of the substrate as in steps 7.2.1 - 7.2.2.

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 100 µL of substrate plus 100 µL of stop solution against a water blank. If the absorbance is greater than 0.1, obtain a new substrate (substrates are not kit specific) so the sensitivity of the assay will not be compromised.

7.9 Place the microtiter plate into a new Ziploc™ bag. Incubate at room temperature for 30 ± 2 minutes. Do not shake during the 30 minutes. Shaking of the plate can result in poor precision and a higher background.

7.10 Remove the plate from the Ziploc™ bag and pipette 100 µL of the **stop solution** as in step 7.2. If the sample is not biohazard, then continue to step 7.11. If the plate contains a virus sample, place an optical adhesive cover (see 4.16) over the plate and the plate is now ready to be read on the plate reader. Place the plate with the optical adhesive cover on it into a Ziploc™ bag. Then place the Ziploc™ bag in a second Ziploc™ bag and decontaminate the bag with Dispatch and then 70% Isopropanol Alcohol. Take the decontaminated bag to the plate reader and carefully remove the plate.

7.11 Read absorbance at 450/650 nm, blanking on the zero standard. Refer to ***SOP 22100 - Operation of the Labsystems iEMS Microtiter Plate Reader/ Dispenser*** for analysis of the data.

NOTE: Read the plates within 30 minutes after adding the stop solution since the color will fade over time. Record read time on Attachment 4.

8.0 Data Analysis and Acceptance Criteria

After the Ascent HEK 293 Host Cell Protein Results (Attachment 2) have been printed out 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 Go to windows “Start.” Select programs and click on “Microsoft Excel.”

8.2 Click on “File” and “Open”. Select “QC Shared on ‘Perfection’ (I:).”

8.3 Click on the “QC Public” folder. Click on the “ELISA Results” folder. Select the specific ELISA test being analyzed; for example, BSA Template, Murine IgG Template, HEK293 Host Cell Protein Template etc.

8.4 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.

8.5 Fill in the “HEK 293 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 > 4 ng/mL.

8.6 Fill in the “Expected” and “Found” Positive Control and the “% CV” from the Ascent ELISA Results. The positive control will be one of the standards that the analyst manually made up and included in the standard curve (see step 6.2.1). The “%CV” should be less than 25%. The “Found” positive control should be 100% ± 30 of what is “Expected”.

8.7 Fill in the “Found” Spiked Buffer Control and the “% CV.” The spiked-buffer control is the buffer that the test article is in, spiked with a known amount of standard (see step 6.1.2). The “%CV” should be less than 25%. The “Found” spike buffer control should be 100% ± 30 of what is “Expected.”

8.8 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%

8.9 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% ± 30.

8.10 The test article must exhibit dilution-corrected analyte concentrations that vary no more than ± 50% between dilutions. Avoid consideration of the dilutional data where the assay value before dilution correction falls below two times the LOQ of the assay (4 ng/mL for the HEK293 HCP assay). The first dilution-corrected concentration exhibiting dilutional linearity is reported. In the example below, the reported result would be 50 ng/mL.

Sample Dilution	Dilution-Corrected Value (ng/mL)	Percent Change in Concentration from the Previous Dilution
neat	100	N/A
1:5	50	50
1:25	45	90
1:50	47	104

8.11 If the criteria in sections 8.9 and 8.10 are not both met, then the assay is invalid.

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 HEK 293 HCP ELISA Results (Attachment 2).

9.2 The lowest dilution with the acceptable criteria listed above can be recorded on the QC Test Request Form.

NOTE: If the results are less than the lowest standard, it is recorded as < 4 ng/mL.

9.3 Record all solution preparation on Form 23103-01 HEK 293 HCP ELISA Preparation Form and include with the QC Test Request form.

10.0 References and Related Documents

10.1 **BDP SOP 22100** *Operation of the Labsystems iEMS Microtiter Plate Reader/ Dispenser*

10.2 **BDP SOP 22702** *Solutions used in Process Analytics*

10.3 **BDP SOP 22923** *Procedure for Safe Handling and Decontamination of Viruses by BDP/BPA and Related Personnel*

10.4 Cygnus Technologies HEK 293 Host Cell Protein ELISA Kit Instructions (Attachment 4).

10.5 Cygnus Suggested Manual Microtiter Plate Washing Procedure.

10.6 Form 23103-01 *HEK 293 Host Cell Protein ELISA Preparation*

11.0 Attachments

11.1 **Attachment 1** Sample ELISA Work List

11.2 **Attachment 2** Sample Ascent HEK 293 Host Cell Protein ELISA Results

11.3 **Attachment 3** Sample Excel Summary Worksheet

11.4 **Attachment 4** *Cygnus Technologies Inc. HEK 293 HCP Kit Insert*

12.0 Change Summary

Section [redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

Attachment 1

Sample ELISA Work List

H	G	F	E	D	C	B	A	↑
200 ng/mL Standard	75 ng/mL Standard	25 ng/mL Standard	12.5 ng/mL Standard	10 ng/mL Standard	4 ng/mL Standard	0 ng/mL Standard	BLANK 0 ng/mL Std	1
200 ng/mL Standard	75 ng/mL Standard	25 ng/mL Standard	12.5 ng/mL Standard	10 ng/mL Standard	4 ng/mL Standard	0 ng/mL Standard	BLANK 0 ng/mL Std	2
					Spiked Buffer Control	Buffer Control	Positive Control	3
					Spiked Buffer Control	Buffer Control	Positive Control	4
		SPIKED SAMPLE	SAMPLE 1:50	SPIKED SAMPLE	SAMPLE 1:5	SPIKED SAMPLE	SAMPLE Undiluted	5
		SPIKED SAMPLE	SAMPLE 1:50	SPIKED SAMPLE	SAMPLE 1:5	SPIKED SAMPLE	SAMPLE Undiluted	6
								7
								8
								9
								10
								11
								12

Assay:

Sample ID:

Date:

Analyst:

Attachment 2

Sample Ascent HEK 293 HCP ELISA Result

Ascent Software Curve Fit1 1/5/06 2:21 PM

Session: [redacted]
Instrument: EMS Reader MF V2.9-0D
User name: [redacted]
Started at: 4/28/05 3:27:27AM
Actual temperature: Amb.temp.

Layout map for calibrators Sheet: Measure1, Assay: Assay1 and for samples Sheet: Measure1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	Blank	Blank		nc	nc		_001	_001		
B	Cal1	Cal1		snc	snc		s001	s001		
C	Cal2	Cal2		neg	neg		_001	_001		
D	Cal3	Cal3		sneg	sneg		s001	s001		
E	Cal4	Cal4		buf	buf					
F	Cal5	Cal5		sbuf	sbuf					
G	Cal6	Cal6		_001	_001					
H	Cal7	Cal7		s001	s001					

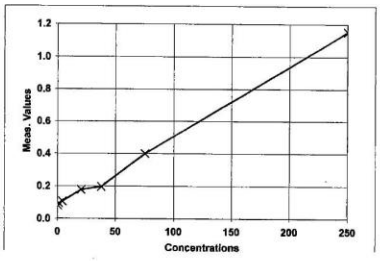
Source data for calibrators Sheet: Measure1, Assay: Assay1 and for samples Sheet: Measure1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	0.073	0.074		0.073	0.070		0.072	0.073		
B	0.080	0.078		0.293	0.274		0.309	0.261		
C	0.097	0.087		0.082	0.075		0.119	0.114		
D	0.106	0.103		0.251	0.213		0.349	0.308		
E	0.183	0.170		0.082	0.081					
F	0.214	0.176		0.287	0.253					
G	0.434	0.363		0.096	0.080					
H	1.143	1.163		0.262	0.229					

Sheet: Measure1, Assay: Assay1

Name	Meas.	Conc.
Cal1	0.080	
	0.078	
	0.079	0.00
Cal2	0.097	
	0.087	
	0.092	1.00
Cal3	0.106	
	0.103	
	0.105	4.00
Cal4	0.183	
	0.170	
	0.177	20.00
Cal5	0.214	

	Conc.	Meas.	CalcConc.	Residual
Cal1	0	0.079	0	0
Cal2	1	0.092	1	0
Cal3	4	0.105	4	0
Cal4	20	0.177	20	0
Cal5	37.5	0.195	37.5	0
Cal6	75	0.399	75	0
Cal7	250	1.153	250	0



Pages: 1/3

Attachment 3
Sample EXCEL Summary Worksheet

Summary of HEK 293 HCP ELISA Results

QC Number: QC-00000 **Analyst:** [redacted] **Date:** 4/19/2010

Sample ID: Project Name **Lot #:** LXX00000

Kit Lot#: 91110 **Expiration Date:** 1/31/2011

HEK 293 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	195.02	190.32	195.02	190.32	**192.67**	**1.72**
5.00	40.04	46.32	200.20	231.60	215.90	10.28
10.00	21.26	19.52	212.60	195.20	203.90	6.03
20.00	11.68	10.58	233.60	211.60	222.60	6.99
40.00	5.33	4.65	213.20	186.00	199.60	9.64

Final Result: **192.67 ng/mL**

Corrected Concentration = Measured Concentration x dilution factor

*n/c = not calculated

Calculated Concentration of "HEK 293 HCP Spiking Solution" (ng/ml): 200.0

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):		37.50	33.14	88.37%	7.88
Spiked Buffer Control	**(ng/mL)**	50.00	52.00	104.00%	8.15

Dilutional Linearity

Dilution Factor		Average Dilution Corrected Concentration	Percent Change in Dilution Corrected Concentration from Previous Value
1.00		192.67	N/A
5.00		215.90	112
10.00		203.90	94
20.00		222.60	109
40.00		199.60	90

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

Attachment 3 (Continued)

Sample EXCEL Summary Worksheet

Spiked Test Article Concentrations (ng/ml; not corrected for dilution):
Acceptable Criteria: %CV <25%

Dilution Factor	Replicate 1	Replicate 2	Average	%CV
1	210.65	206.54	**208.60**	**1.39**
5	85.66	86.34	86.00	0.56
10	56.30	60.54	58.42	5.13
20	60.48	57.89	59.19	3.09
40	56.23	52.47	54.35	4.89

Percent Spike Recovery of Test Article:
Acceptable Criteria: Average Recovery (100% ± 30) and %CV <25%

Dilution Factor	Replicate 1	Replicate 2	Average	%CV
1	128.77	127.60	**128.19**	**0.65**
5	111.26	103.20	107.23	5.32
10	80.71	91.80	86.26	9.09
20	103.44	99.91	101.68	2.45
40	104.47	97.97	101.22	4.54

Percent Spike Recovery = (((Spiked Conc x 0.050 mL)-(Unspiked Conc x 0.0375 mL))/(Spike Conc x 0.0125 mL)) x 100%

Analyst/Date: _____

Reviewed By/Date: _____

Attachment 4

CYGNUS TECHNOLOGIES
part of Maravai LifeSciences

HEK 293 Host Cell Proteins

Immunoenzymetric Assay for the Measurement of HEK 293 Host Cell Proteins Catalog # F650R

Intended Use

This kit is intended for use in determining the presence host cell protein impurities in products manufactured by expression in HEK 293 host cells. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals. Users should qualify this assay for use with their product samples. The "R" designation in the Catalog # of F650R and in the component product numbers indicates that this kit incorporates a replacement antibody for the antibody used in F650 kits prior to March 2017. This new antibody was prepared using the same immunogens and affinity purified in the same way as the original F650 kit. As such the broad reactivity and specificity should be very similar. Those labs previously using the original F650 kit and antibodies should perform equivalency, bridging, or re-qualification studies as deemed necessary to determine if there are significant quantitative differences in HCP levels in their samples. As part of the introduction of this new antibody we have included other minor reagent changes to improve the low end sensitivity and to extend the shelf life of the kit components.

Summary and Explanation

Expression of vaccines or other therapeutic proteins in HEK 293 cells is a widely used procedure to obtain sufficient and cost effective quantities of a desired protein or virus. Many of these products are intended for use as therapeutic agents in humans and animals and as such must be highly purified. The manufacturing and purification process of these products leaves the potential for impurities by host cell proteins from HEK 293 cells. HCP impurities can result in adverse toxic or immunological reactions and thus it is desirable to reduce host cell impurities to the lowest levels practical. The polyclonal antibodies used in this kit have been generated against and affinity purified using a mild lysate of HEK 293 cells to obtain HCPs typically encountered in initial product recovery steps.

Special procedures were utilized in the generation of these antibodies to insure that low molecular weight and less immunogenic impurities 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 impurities as well as in routine final product release testing when comprehensively characterized using samples from your process. The orthogonal methods of Antibody Affinity Extraction (AAE) and Western blot were used to determine reactivity to individual HCPs in a mild lysate of HEK 293. AAE indicated reactivity to ~70% of the individual 2D PAGE fractionated spots presumed to be HCP. In terms of the total mass of HCP, the anti-HEK 293 antibody reacts to those HCPs that represent >90% of the total HCP mass in the lysate sample.

Cygnus has qualified this assay using actual in-process samples from 6 different drug products including virus, vaccines and therapeutic proteins. The assay was broadly reactive to the vast majority of total upstream HCPs in all cell lines and had the sensitivity to detect HCP in all final drug substances. Despite such qualitative indications this kit may be useful for other products expressed in HEK 293, it is required that each end user qualify the antibody and assay for their analytical needs. Provided the kit can be satisfactorily qualified for your samples, the application of a more process specific assay may not be necessary. If your qualification 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. A process specific 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 impurities 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 sensitivity, however, such an assay runs the risk of being too specific in that it may fail to detect new or atypical impurities that might result from some process irregularity or change. For this reason it is recommended that a broadly reactive "generic" or "platform" 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 offers custom antibody and assay development services using our proven technologies.

Attachment 4 (Continued)

Principle of the Procedure

The HEK 293 Host Cell Protein assay is a two-site immunoenzymetric assay. Samples containing HEK 293 cell proteins are reacted in microtiter strips coated with an affinity purified capture antibody. An HRP labeled anti-HEK 293 antibody is reacted simultaneously, forming a sandwich complex of solid phase antibody-HEK 293 HCP-enzyme labeled antibody. The microtiter strips are then washed to remove any unbound reactants. After the washes, the substrate tetramethylbenzidine is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of HEK 293 Host cell proteins present.

Reagents & Materials Provided

Component	Product #
Anti-HEK 293:HRP Affinity purified rabbit antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F651R
Anti-HEK 293 coated microtiter strips Blend of goat and rabbit antibodies coated on 12x8 well strips in a bag with desiccant.	F652R*
HEK 293 HCP Standards HEK 293 HCPs in Catalog # I094 with preservative. Standards at 0, 4, 10, 25, 75, 200ng/mL, 1 mL/vial.	F653R
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 # F652R.

Storage & Stability

- If the kit will not be utilized within 1 week after receipt it is recommended to store the standards at -10°C to -30°C. All other reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- 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.

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
- Orbital Microtiter plate shaker (400 - 600 rpm)
- Sample Diluent (recommended Cat #I094)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H_2SO_4. 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

1. 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 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 0.200, 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 with HCP greater than 800µg/mL may give absorbances less than the 200ng/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 dilution linearity/parallelism as evidenced by an apparent increase in dilution corrected HCP concentration with increasing dilution. If a hook effect is possible, samples should also be assayed diluted. If the HCP concentration of the undiluted or less diluted sample is less than a more diluted sample, this may be indicative of the hook effect.

Attachment 4 (Continued)

Such samples should be diluted at least to the minimum required dilutions (MRDs) as established by your qualification 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 # I094 available in 100mL, 500mL, or 1 liter bottles. The previous F650 kit used Cat # I028 as the diluent. I028 may give a slight difference in low end non-specific binding as compared to I094. This should only affect interpolated values very near the LLOQ of the assay. If this is not deemed significant you may use I028 as a sample diluent. As the sample is diluted in I094, 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 200ng/mL standard, as described in the "Limitations" section below.

Limitations

- Before relying exclusively on this assay to detect host cell proteins, each laboratory should qualify that the kit antibodies and assay procedure yield acceptable coverage, dilutional linearity, accuracy, and precision. A suggested protocol for this qualification can be obtained from our Technical Services Department or our web site. It is advised to use orthogonal methods to determine the anti-HCP antibody reactivity to individual HCPs from your strain and process. Cygnus recommends the use of AAE over 2D WB because AAE is much more sensitive and specific than 2D WB. At least 2 samples should be subjected to AAE; a harvest-like sample to demonstrate coverage to all upstream HCPs and a downstream sample to show coverage to those HCPs that co-purify with your product. Cygnus Technologies offers the AAE characterization service for your samples and can also advise or perform other assay qualification services for you.
- The standards used in this assay are comprised of HEK 293 HCPs solubilized by mechanical disruption and detergent. AAE analysis of the antibodies used in this kit demonstrates that they recognize the majority of distinct PAGE separated proteins seen using a sensitive protein staining method like silver stain or colloidal gold. Because the vast majority of HCPs will be conserved among all strains of HEK 293 this kit should be adequately reactive to HCPs from your strain
- 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 200ng/mL standard, 1 part to 3 parts of the matrix containing no or very low HCP impurities. This diluted standard when assayed as an unknown, should give an added HCP value in the range of 40 to 60ng/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 suggested assay protocol takes approximately 2.5 hours to complete. With good technique and precision the assay can yield sensitivity lower limit of quantitation of <2ng/mL. 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 Technical Services for input on the best way to achieve your desired goals.
- The protocol specifies use of an approved orbital microtiter plate shaker for the immunological step. 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. In addition, a video demonstration of proper plate washing technique is available in the 'Technical Help' section of our web site.
- All standards, controls, and samples should be assayed at least in duplicate.

Attachment 4 (Continued)

- 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.
- The 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.
- 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.**
- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Pipette 100µL of anti-HEK 293:HRP (#F651R) into each well.

2. Pipette 50µL of standards (#F653R), controls and samples into wells indicated on work list.

3. Cover & incubate on orbital plate shaker at 400 - 600 rpm for 2 hours at room temperature, 24°C ± 4°C.

4. 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.

5. Pipette 100µL of TMB substrate (#F005).

6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.

7. Pipette 100µL of Stop Solution (#F006).

8. Read absorbance at 450/650nm.

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 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 15% for samples in the range of 10-200ng/mL. CVs for samples <10ng/mL may be greater than 15%.
- It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

Example Data

Well #	Contents	Abs. at 450nm-650nm	Mean Abs.
A1	0ng/mL	0.092	0.086
A2	0ng/mL	0.079	
B1	4ng/mL	0.131	0.135
B2	4ng/mL	0.139	
C1	10ng/mL	0.218	0.217
C2	10ng/mL	0.215	
D1	25ng/mL	0.410	0.408
D2	25ng/mL	0.405	
E1	75ng/mL	1.015	0.993
E2	75ng/mL	0.971	
F1	200ng/mL	1.872	1.778
F2	200ng/mL	1.685	

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. This qualification is generic in nature and is intended to supplement but not replace certain user and product specific qualification 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 dilution 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. Orthogonal antibody characterization studies as discussed in the above "Summary and Explanation" and "Limitation" sections should also be performed. A more detailed discussion of recommended user qualification protocols can be obtained by contacting our Technical Services Department or on-line at our website.

Attachment 4 (Continued)

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 ~1 ng/mL.
The lower limit of quantitation (**LOQ**) is defined as the lowest concentration, where concentration coefficients of variation (CVs) typically are <20%. The LOQ is ~2 ng/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=10 assays) precision were determined on 3 pools with low (10ng/mL), medium (25ng/mL), and high concentrations (75ng/mL). The %CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	8.2%	8.6%
Medium	4.3%	10.8%
High	8.8%	6.1%

Recovery / Interference Studies

Various buffer matrices have been evaluated by adding known amounts of HEK 293 HCPs used to make the standards in this kit. Because this assay is designed to minimize matrix interference most of these buffers yielded acceptable recovery (defined as between 80-120%). In general extremes in pH (<6.0 and >8.5) as well as certain detergents can cause under-recovery. Organic solvents and high salt concentration can also interfere. In some cases very high concentrations of the product protein may also cause a negative interference in this assay. Each user should demonstrate that their sample matrices and product itself yield accurate recovery. Such an experiment can be performed by diluting the 200ng/mL standard provided with this kit into the sample in question. For example, we suggest adding 1 part of the 200 ng/mL standard to 3 parts of the test sample. This yields an added spike of 50ng/mL. Any endogenous HEK 293 HCPs from the sample itself determined prior to spiking and corrected for by the 20% dilution of that sample can be subtracted from the value determined for the spiked sample. The added spike and recovery should be within allowable limits e.g. 80% to 120%. Should you have any problems achieving adequate spike and recovery data you are strongly urged to contact our Technical Services Department for recommendations on how to overcome sample matrix interference.

Hook Capacity

Increasing concentrations of HCPs from 200 to 600,000 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration that will give an absorbance reading less than the 200 ng/mL standard was >150µg/mL.

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the extraction of Host cell DNA. The following kits are available:

- Human Host cell DNA detection by Picogreen:

  Cat # D160W, DNA Extraction Kit in 96 deep well plate
  Cat # D160T, DNA Extraction Kit in microfuge tubes

- Residual Host Cell DNA extraction:

  Cat # D100W, DNA Extraction Kit in 96 deep well plate
  Cat # D100T, DNA Extraction Kit in microfuge tubes

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

www.cygnustechnologies.com
Cygnus Technologies, LLC
4332 Southport Supply Rd. SE
Southport, NC 28461 USA
Tel: 910-454-9442
Fax: 910-454-9443
Email: techsupport@cygnustechnologies.com