Biopharmaceutical Development Program

Standard Operating Procedure

Title: Quantitation of *E.coli* Host Cell Protein Using the Cygnus Inc. ELISA Kit

SOP Number: 22104 **Revision Number: 05**

Supersedes: Revision 04 **Effective Date: OCT 21 2011**

Originator/Date: [redacted]

Approval/Date:

Approval/Date:

Table of Contents

1.0 Purpose

This SOP describes the use of the Cygnus, Inc. ELISA Kit to determine the presence of *E. coli* protein contamination in products manufactured in *E. coli* host cells.

2.0 Scope

This SOP applies to Process Analytics (PA) personnel who will perform the *E. coli* Host Cell Protein ELISA.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

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.

3.0 Authority and Responsibility

3.1 The Director, Process Analytics (PA) has the authority to define this procedure.

3.2 PA is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA personnel are responsible for the performance of this procedure.

3.4 PA is responsible for reviewing the data and documenting of the results of this procedure.

3.5 BQA is responsible for quality oversight of this procedure.

4.0 Materials and Equipment

4.1 *E. coli* Host Cell Protein ELISA Kit, BDP PN 30404 Cygnus Technologies, Catalog Number F410.

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

4.3 Calibrated multi-channel pipettor.

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, VWR, or BDP approved equivalent.

4.6 Microtiter Plate Shaker, VWR Catalog Number 57019-600, or equivalent.

4.7 Ziploc™ Bags, 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 Direct-Q water or equivalent.

4.11 1 Liter Graduated Cylinder for 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 Labsystems iEMS Microtiter Plate Reader MF with Ascent software version 2.4.2, Model Number 1401, BDP MEF 66160.

5.0 General Comments

5.1 Pipetting accuracy and reproducibility are critical for the success of this assay.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

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

5.3 Avoid contamination of the workspace when handling *E. coli* standards, ejecting pipette tips, etc. Pipette tips possessing an aerosol barrier must be used.

5.4 Good organization and attention to detail is essential to avoid confusion of sample identities and data.

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

6.0 Preparation

Note: The reagents are located in the 2-8°C refrigerator. Bring all reagents to ambient temperature (up to one hour). All standards, controls, and samples must be assayed in duplicate. The total volume of the samples should account for this.

6.1 Sample Preparation: Range of Assay = 1 -100 ng/mL

6.1.1 Prepare the test article immediately before use, neat and at 1:5 and 1:50 dilution using the Cygnus, Inc. diluent (see 4.2). If the absorbance results indicate that the value for the 1:50 dilution is > 100 ng/mL, repeat the ELISA assay with appropriately-diluted samples. An example for making dilutions is the following.

For duplicate measurements of spiked and un-spiked diluted test article (a total of four 25 µL aliquots), add 100 µL test article to 400 µL diluent in a microcentrifuge tube to give a 1:5 dilution.

A 1:50 dilution can be made up in a microcentrifuge tube 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:

A spiked test article can be made up directly into the microtiter strip well. For a spiked concentration of 25 ng/mL host cell protein, do a 4x dilution using the 100 ng/mL Cygnus, Inc. *E. coli* standard. For example, add 6.25 µL of the 100 ng/mL standard directly into the well containing 18.75 µL of the test article prepared in step 6.1.1. This will need to be done in duplicate. 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.

A spiked-buffer control sample can be made up directly into the microtiter strip well. For a spike-buffer control, add 6.25 µL of the 100 ng/mL standard directly into the well containing 18.75 µL of the buffer provided by the requestor. This produces a 25 ng/mL concentration of HCP in the spiked-buffer control sample. This will need to be done in duplicate. 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

Prepared standards at 0, 1, 3, 12, 40, and 100 ng/mL *E. coli* Host Cell Protein are included in the Cygnus *E. coli* HCP kit.

6.3 Positive Control Preparation

6.3.1 A 20 ng/mL positive control is prepared directly into the microtiter strip well. To make the 20 ng/mL positive control, do a 1:1 dilution of the 40 ng/mL standard. Add 12.5 uL of the 40 ng/mL standard to 12.5 uL of the diluent directly into the microtiter strip well. This will need to be done in duplicate. 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.

6.4 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 Direct-Q water. Log the wash solution in the Process Analytics Solution Logbook. Label the solution bottle with the BQC number, initials, date prepared, and expiration date. Refer to **SOP 22702, *Solutions Used in Process Analytics***.

7.0 Procedure

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

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 25 μL of standards, controls and samples into wells indicated on the ELISA Work list (Attachment 1). Assay all standards, controls and samples in at least duplicate.

7.3 Pipette 100 μL of anti-*E.coli*: HRP into each well.

7.3.1 Pour contents of anti-*E.coli*: HRP bottle into a new reagent reservoir (4.8).

7.3.2 Use a multi-pipettor to fill rows of wells. Get fresh pipette tips with each row that is filled.

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

7.5 Transfer the plate to the microtiter plate shaker (4.6) and incubate for 90 ± 2 minutes at a setting of "2" (180 rpm).

7.6 Using a manual microtiter plate washing 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.

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 you rapidly

accelerate your arm and hand downward. Abruptly stop your arm, causing the liquid to be forced from the strips into the sink. Repeat the removal motion a second time.

7.6.2 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 as described in steps 7.6.1.

Blot and strike the plate as follows. 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 30 seconds. Strike the plate hard 4 times over unused areas of the paper. Do not be afraid to strike vigorously. Anything short of breaking the strip-holder or strips is not too hard.

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^{o} in your hand between each strike. This rotation ensures that the ends of the plate receive on average the same energy and impact. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding TMB substrate.

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 and repeat step 7.6.3. Wells are now ready to have the substrate added to them.

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

7.7 Pipette 100 uL of the substrate as in steps 7.3.1-7.3.2.

Note: If the substrate has a distinct blue color prior to the assay, it may have been contaminated. If this appears to be the case, read 100 uL of substrate plus 100 uL of stop against water blank. If the absorbance is greater than 0.2, obtain a new substrate (the substrate is not kit specific) 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 ± 2 minutes. Do not shake.

7.9 Remove the plate from the Ziploc™ bag. Pipette 100 uL of stop solution as in steps 7.3.1-7.3.2.

7.10 Read absorbance at 450/630 nm, blanking on the zero standard. Refer to **SOP 22100, *Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser,*** for analysis of the data.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

8.0 Data Analysis and Acceptable Criteria

After the Ascent *E. coli* Host Cell Protein Results (Attachment 2) have been printed from the ELISA run, the results will need to be entered manually 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 the windows "Start." Select programs and click on "Microsoft Excel."

8.2 Click on "My Computer." Select "scidata on “fr-s-bdp-vlan’ (s:)."

8.3 Click on the "PA" folder. Click on the “ELISA Results” folder. Select the specific ELISA Test being analyzed; for example, BSA Template, Murine IgG Template, *E.coli* 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 "*E.coli* HCP Concentration" section with appropriately 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.

8.6 Fill in the "Expected" and "Found" Positive Control and the "% CV" from the Ascent ELISA Results. The positive control will be the 1:1 dilution of the 40 ng/mL standard as described in 6.3. The "%CV" should be less than 25%. The “found” positive control should be 100% $\pm$ 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 control should be 100% $\pm$ 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% $\pm$ 30.

8.10 The test article must exhibit dilution-corrected analyte concentrations that vary no more than ± 50% between dilutions. Avoid consideration of dilutional data where the assay value before dilution correction falls below two times the LOQ of the assay (1 ng/mL for the *E coli* HCP assay). The first dilution-corrected concentration exhibiting dilutional linearity is reported. In the example below, the reported result would be 50 ng/mL.

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Sample Dilution	Dilution-Corrected Value (ng/mL)	Percent Change in Concentration from 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 the assay is invalid.

9.0 Documentation

9.1 Print 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 *E.coli* HCP ELISA Results (Attachment 2).

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

Note: If less than the lowest standard, it is recorded as < 3 ng/mL.

9.3 Record the solution preparation on Form 22104-01 *E.coli* HCP ELISA Preparation Form (Attachment 4) and included with the QC Test Request.

10.0 References and Related Documents

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

10.2 **SOP 22702** *Solutions Used in Process Analytics*

10.3 Cygnus Technologies *E. coli* Host Cell Protein Kit Instructions (Attachment 5).

10.4 Cygnus Suggested Manual Microtiter plate Washing Procedure.

11.0 Attachments

11.1 **Attachment 1** Sample ELISA Work List

11.2 **Attachment 2** Sample of Ascent *E.coli* Host Cell Protein ELISA Results

11.3 **Attachment 3** Sample Excel Summary Worksheet

11.4 **Attachment 4** Form 22104-01, E.coli HCP ELISA Preparation

11.5 **Attachment 5** Cygnus Technologies Inc., *E.coli* HCP ELISA Kit Insert

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

Attachment 1
Sample ELISA Work List

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

Assay: E.coli HCP ELISA

Sample ID:

Date:

Analyst:

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

Attachment 2
Sample of Ascent *E.coli* Host Cell Protein ELISA Results

Ascent Software Curve Fit1 11/19/08 5:32 PM

Session: C:\ASCENT\K102907.SEE
Instrument: EMS Reader MF V2.9-0D
User name: [redacted]
Started at: 10/29/07 3:58:40PM
Actual temperature: Amb.temp.

Layout map for calibrators Sheet: Precalc1, Assay: Assay1 and for samples Sheet: Precalc1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	Blank	Blank		Pos	Pos		CAT	CAT		
B	Cal1	Cal1					sCAT	sCAT		
C	Cal2	Cal2					CAT	CAT		
D	Cal3	Cal3					sCAT	sCAT		
E				CAT	CAT					
F	Cal4	Cal4		sCAT	sCAT					
G	Cal5	Cal5		CAT	CAT					
H	Cal6	Cal6		sCAT	sCAT					

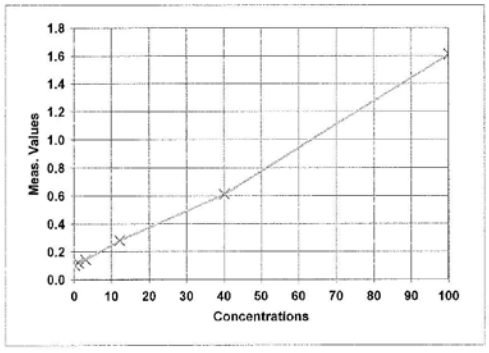
Source data for calibrators Sheet: Precalc1, Assay: Assay1 and for samples Sheet: Precalc1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	0.102	0.101		0.102	0.098		0.169	0.172		
B	0.101	0.103					0.301	0.275		
C	0.121	0.117					0.184	0.156		
D	0.144	0.137					0.328	0.324		
E				0.119	0.106					
F	0.291	0.269		0.247	0.239					
G	0.644	0.580		0.128	0.120					
H	1.642	1.583		0.278	0.286					

Sheet: Precalc1, Assay: Assay1

Name	Meas.	Conc.
Cal1	0.101	
	0.103	
	0.102	0.00
Cal2	0.121	
	0.117	
	0.119	1.00
Cal3	0.144	
	0.137	
	0.141	3.00
Cal4	0.291	
	0.269	
	0.280	12.00
Cal5	0.644	
	0.580	

	Conc.	Meas.	CalcConc.	Residual
Cal1	0	0.102	0	0
Cal2	1	0.119	1	0
Cal3	3	0.141	3	0
Cal4	12	0.280	12	0
Cal5	40	0.612	40	0
Cal6	100	1.613	100	0



Pages: 1/3

Attachment 3
Sample Excel Summary Worksheet

Summary of *E. coli* HCP ELISA Results

QC Number: QC-00000 **Analyst:** [redacted] **Date:** 4/2/2009

Sample ID: Project Name **Lot #:** LXX0000

Kit Lot#: 27019 **Expiration Date:** 12/31/2009

I. E.coli HCP Concentration (ng/ml)

Acceptable Criteria: %CV <25% for samples >3ng/mL

Dilution Factor	Measured Concentration		Corrected Concentration		Average	%CV
	Replicate 1	Replicate 2	Replicate 1	Replicate 2		
1.00	29.69	26.60	29.69	26.60	28.15	7.76
5.00	12.41	13.47	62.05	67.35	**64.70**	**5.79**
10.00	4.69	5.17	46.90	51.70	49.30	6.88
20.00	3.19	3.05	63.80	61.00	62.40	3.17
40.00	1.43	0.80	57.20	32.00	44.60	39.95

Final Result: **64.70 ng/mL**

Corrected Concentration = Measured Concentration x dilution factor

*n/c = not calculated

Calculated Concentration of "*E. coli* 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	20.01	100.05%	0.61
Spiked Buffer Control (ng/mL)	25.00	25.00	100.00%	0.00

Dilutional Linearity

Dilution Factor		Average Dilution Corrected Concentration	Percent Change in Dilution Corrected Concentration from Previous Value
1.00		28.15	N/A
5.00		64.70	230
10.00		49.30	76
20.00		62.40	127
40.00		44.60	71

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

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Attachment 3 (Continued)

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

Dilution Factor	Replicate 1	Replicate 2	Average	%CV
1	44.48	41.70	43.09	4.56
5	35.44	33.73	34.59	3.50
10	30.64	26.41	28.53	10.49
20	32.40	28.41	30.41	9.28
40	27.79	27.70	27.75	0.23

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

Dilution Factor	Replicate 1	Replicate 2	Average	%CV
1	88.85	87.00	87.93	1.49
5	104.53	94.51	99.52	7.12
10	108.49	90.13	99.31	13.07
20	120.03	104.49	112.26	9.79
40	106.87	108.40	107.64	1.01

Percent Spike Recovery = (((Spiked Conc x 0.025 mL)-(Unspiked Conc x 0.01875 mL))/(Spike Conc x 0.00625 mL)) x 100%

Analyst/Date: _____

Reviewed By/Date: _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

Attachment 4

NCI-Frederick
Form No.: 22104-01
SOP No.: 22104
Revision 05:

E.COLI HCP ELISA PREPARATION

QC Number: _____ **Operator**:_____ **Date**:_____

Plate Reader MEF Number: _____ Calibration Due Date: _____
Diluent:

Name: _____ Lot#_____ Exp. Date: _____

Test sample Dilutions:

Test Sample #1
Name:_____ Appearance: _____
Lot #_____
Protein Concentration: _____mg/mL

Initial dilution:_____
Volume of diluent:_____ µL
Volume of test sample:_____ µL

Second dilution:_____
Volume of diluent:_____µL
Volume of initial diluted test sample_____µL

Third Dilution _____
Volume of Diluent: _____µL
Volume of Second Diluted sample _____µL

Fourth dilution:_____
Volume of diluent:_____µL
Volume of Third diluted test sample_____µL

Fifth Dilution _____
Volume of Diluent: _____µL
Volume of Fourth Diluted sample _____µL

Test sample #2
Name:_____ Appearance: _____
Lot #_____
Protein Concentration: _____mg/mL

Initial dilution:_____
Volume of diluent:_____ µL
Volume of test sample:_____ µL

Second dilution:_____
Volume of diluent:_____µL
Volume of initial diluted test sample_____µL

Third Dilution _____
Volume of Diluent: _____µL
Volume of Second Diluted sample _____µL

Fourth dilution:_____
Volume of diluent:_____µL
Volume of Third diluted test sample_____µL

Fifth Dilution _____
Volume of Diluent: _____µL
Volume of Fourth Diluted sample _____µL

Test sample #3
Name:_____ Appearance: _____
Lot #_____
Protein Concentration: _____mg/mL

Initial dilution:_____
Volume of diluent:_____ µL
Volume of test sample:_____ µL

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Attachment 4 (Continued)

NCI-Frederick
Form No.: 22104-01
SOP No.: 22104
Revision 05:

E.COLI HCP ELISA PREPARATION

Second dilution:_____
Volume of diluent:_____µL
Volume of initial diluted test sample_____µL

Third Dilution _____
Volume of Diluent: _____µL
Volume of Second Diluted sample _____µL

Fourth dilution:_____
Volume of diluent:_____µL
Volume of Third diluted test sample_____µL

Fifth Dilution _____
Volume of Diluent: _____µL
Volume of Fourth Diluted sample _____µL

Positive Control
Preparation of Positive Control
Concentration: _____ ng/mL
Std: _____µL
Sample Dilution Buffer:_____µL

Spiked Buffer Control
Preparation of Spiked Buffer Control
Concentration: _____ ng/mL
Std: _____µL
Sample Buffer:_____µL

Template Procedure

anti-E.coli: HRP
Lot# _____
Expiration Date:_____
Volume of anti-E.coli: HRP added to wells:_____
Incubation Time:_____
Washing times:_____

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

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

Data Storage
File Name:_____

Performed by/Date: _____

Reviewed by/Date: _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

Attachment 5
Cygnus Technologies Inc. *E.coli* HCP ELISA Kit Insert

Cygnus Technologies, Inc.
Revision #6-07

E. coli Host Cell Proteins

Immunoenzymetric Assay for the Measurement of *E. coli* Host Cell Proteins Catalog # F410

Intended Use

This kit is intended for use in determining the presence of *E. coli* host cell protein contamination in products manufactured by recombinant expression in *E. coli*. The kit is for Research and Manufacturing Use Only and is not intended for diagnostic use in humans or animals.

Summary and Explanation

Recombinant expression in *E. coli* is a relatively simple and cost effective method for production of certain proteins and pDNA. 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 contamination by host cell proteins (HCPs) from *E. coli*. 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 widely accepted due to their specificity and sensitivity. While Western blot is a powerful 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 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, highly sensitive, objective, and semi-quantitative ELISA is a powerful method to aid in optimal purification process development, process control, and in 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 against and affinity purified using a blended lysate of *E. coli* from a variety of strains including the commonly used DH5α and BL21 strains. This relatively mild lysing procedure is intended to obtain HCPs typically encountered in initial product recovery steps, such as clarification of conditioned media when the product is secreted, or after osmotic shock or mild detergent and mechanical disruption, to obtain inclusion bodies and other intracellular proteins. 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 testing.

Because of the high sensitivity and broad reactivity of the antibodies, this generic kit has been successfully validated for testing of final product HCPs in many different products regardless of growth and purification process. When the 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 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 suitability of this kit for a given sample type and product must be determined and validated experimentally by each laboratory. 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 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.

Principle of the Procedure

The *E. coli* assay is a two-site immunoenzymetric assay. Samples containing *E. coli* HCPs are reacted with a horseradish peroxidase (HRP) enzyme labeled anti-*E.*

Attachment 5 (Continued)

coli antibody simultaneously in microtiter strips coated with an affinity purified capture anti-*E. coli* 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, tetramethyl benzidine (TMB) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of *E. coli* HCPs present.

Reagents & Materials Provided

Component	Product #
Anti-*E. coli*:HRP Affinity purified antibody conjugated to HRP in a protein matrix with preservative, 1x12mL	F411
Anti-*E. coli* coated microtiter strips 12x8 well strips in a bag with desiccant	F412
***E. coli* HCP Standards** Solubilized *E. coli* HCPs in bovine albumin with preservative. Standards at 0, 1, 3, 12, 40, & 100ng/mL, 1 mL/vial	F413
TMB Substrate 3,3',5,5' Tetramethyl benzidine, 1x12mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative, 1x50mL	F004
Stop Solution 0.5N sulfuric acid, 1x12mL	F006

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 0.2.
* Reconstituted wash solution is stable until the expiration date of the kit.

Materials & Equipment Required But Not Provided

Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 630nm.
(If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)
Pipettors - 25µL and 100µL
Repeating or multichannel pipettor - 100µL
Microtiter plate rotator (150 - 200 rpm)
Sample Diluent (recommended Cat # I028)
Distilled water
1 liter wash bottle for diluted wash solution

Precautions

For research or manufacturing use only.

Stop reagent is 0.5N 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, and 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 manual 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 minus a substrate blank is greater than 0.300, evaluate plate washing procedure for proper performance.

2. High Dose Hook Effect 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 the sample. Samples greater than 200 µg/mL may give absorbances less than the 100 ng/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 undiluted sample may be lower than the highest standard in the kit, however these samples will fail to show acceptable dilutional recovery/parallelism as evidenced by an apparent increase in HCP concentration with increasing dilution. High Dose Hook is 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 dilution (MRD) where the dilution adjusted value remains essentially constant. 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

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

Attachment 5 (Continued)

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 *E. coli* HCPs solubilized by mechanical disruption and detergent. Western blot analysis of the antibodies used in this kit demonstrates that they recognize the majority of distinct PAGE separated bands seen using a sensitive protein staining method like silver stain or colloidal gold. Because the majority of HCPs will show sufficient antigenic conservation among all strains of *E. coli* this kit should be adequately reactive to HCPs from your strain. The antibodies used in this kit were generated against HCPs obtained predominately from DH5α and BL21 strains but they have been shown to react with the majority of HCP from all strains of *E. coli* tested. However, there can be no guarantee that this assay will detect all proteins or protein fragments from your process. In recognition of this potential limitation, we suggest reporting unknown samples in arbitrary dose units such as "ng/mL or parts per million of total immuno-reactive HCP equivalents".

* 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 protein or other components in the sample matrix may result in either positive or negative interference in this assay. It is advised to test all sample matrices for interference by diluting the 100 ng/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 a value of 15 to 30 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 (NaN_3) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

Assay Protocol

*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, users are advised to contact our 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. 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 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 ~630nm 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.
* 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 the assay it may have been contaminated. If the absorbance of 100µL of substrate plus 100µL of stop against a water blank is greater than 0.2 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.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

Attachment 5 (Continued)

Assay Protocol

1. Pipette 25µL of standards, controls and samples into wells indicated on work list.

2. Pipette 100µL of anti-*E. coli*:HRP into each well.

3. Cover & incubate on rotator at ~ 180rpm for 90 minutes at room temperature, 22°C ± 4°.

4. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding well from a squirt bottle or by pipeting in ~350 µL. Dump and bang 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 TMB substrate.

5. Pipette 100µL of substrate.

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

7. Pipette 100µL of Stop Solution.

8. Read absorbance at 450/630nm blanking on the Zero standard.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL "total immuno-reactive HCP equivalents" (See Limitations section above). 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.

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL HCP equivs.
1A	Zero Std	0.066		
1B	Zero Std	0.068	0.067	
1C	1ng/mL	0.099		
1D	1ng/mL	0.098	0.099	
1E	3ng/mL	0.155		
1F	3ng/mL	0.153	0.154	
1G	12ng/mL	0.396		
1H	12ng/mL	0.393	0.395	
2A	40ng/mL	1.190		
2B	40ng/mL	1.179	1.185	
2C	100ng/mL	2.481		
2D	100ng/mL	2.505	2.493	
2E	sample A	0.733		
2F	sample A	0.740	0.737	24.1
2G	sample B	0.152		
2H	sample B	0.151	0.152	2.9

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 < 0.2.

- 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. This validation is generic in nature and is intended to supplement but not replace certain user and product specific 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.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

Attachment 5 (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 ~0.2 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.

Precision

Both intra (n=20 replicates) and inter-assay (n=5 assays) precision were determined on 3 pools with low (~3ng/mL), medium (~12ng/mL), and high concentrations (~40ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	3.3	7.7
Medium	1.9	5.5
High	2.7	6.5

Specificity/Cross-Reactivity

Western blot and ELISA analysis against several strains of *E. coli* (DH5α., BL21, JM109, TOP10F, K12, & MC1061) indicate that most of the proteins are conserved among all strains. Thus, this assay should be useful for detecting HCP's from other *E. coli* cell lines. Cross reactivity 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.

Recovery/ Interference Studies

Various buffer matrices were evaluated by adding known amounts of *E. coli* HCP preparation 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%. The standards used in this kit contain 8mg/mL of bovine serum albumin intended to simulate non-specific protein affects of most sample proteins or pDNA products. However very high concentrations of some products (often in the 2-5 mg/mL range) may interfere in the accurate measurement of HCP's. In general, extremes in pH (<5.0 and >8.5), high salt concentration, high polysaccharide concentrations, 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.

Hook Capacity

Increasing concentrations of HCPs > 100 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 100 ng/mL standard was >200 µg/mL.

Ordering Information/ Customer Service

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

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