



Standard Operating Procedure

Biopharmaceutical Development Program

Title: Quantitation of Residual BSA Using the Cygnus Inc. ELISA Kit

SOP Number: 22119

Revision Number: 03

Supersedes: Revision 02

Effective Date: OCT 25 2011

Originator/Date:

Approval/Date:

Approval/Date:

Table of Contents

- 1.0 Purpose
- 2.0 Scope
- 3.0 Authority and Responsibility
- 4.0 Supplies and Reagents
- 5.0 General Comments
- 6.0 Preparation
- 7.0 Procedure
- 8.0 Data Analysis and Acceptable Criteria
- 9.0 Documentation
- 10.0 References and Related Documents
- 11.0 Attachments

1.0 Purpose

This Standard Operating Procedure (SOP) describes the use of the Cygnus, Inc. ELISA kit for residual Bovine Serum Albumin (BSA) quantitation.

2.0 Scope

This SOP applies to PA personnel who will perform the BSA ELISA.

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 personnel are responsible for performance of this procedure.
- 3.3 PA is responsible for reviewing the data and documentation of the results of this procedure.
- 3.4 BQA is responsible for quality oversight of this procedure.

4.0 Supplies and Reagents

- 4.1 *Bovine Serum Albumin* ELISA kit, BDP PN 30125, Cygnus Technologies, Catalog Number F030.
- 4.2 BSA Sample Diluent, BDP PN 30403 Cygnus Technologies, Catalog Number F031A.
- 4.3 Calibrated multi-channel pipettor.
- 4.4 Calibrated pipettors 2-200 μ L and 100-1000 μ L.
- 4.5 Aerosol Barrier Pipet tips, 2-200 μ L, BDP PN 20673 and 1-1000 μ L, BDP PN 20769, VWR, or BDP approved equivalent.
- 4.6 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, BioWhittaker, BDP PN 20270, or BDP approved equivalent.
- 4.9 Microcentrifuge tubes 0.5mL, BDP PN 21369, or BDP approved equivalent.
- 4.10 DirectQ water, or BDP approved 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 VWR, 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.
- 5.2 Use a new pipette tip for each pipetting procedure (between dilutions).
- 5.3 This kit is a very sensitive assay for BSA. It is very important to use extreme care to avoid contamination of the reagents in this kit with external sources of BSA. Pipette tips possessing an aerosol barrier must be used. Use pipettes that are designated for BSA ELISA ONLY.

- 5.4 Good organization and attention to detail are 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, high salt concentration, extremes of pH (< 6.0 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 will be assayed in duplicate. The total volume of the samples must take this into account.

6.1 Sample Preparation: Linear Range of the Assay =0-32 ng/mL.

- 6.1.1 Prepare the test article immediately before use, undiluted and at 1:5 and 1:50 dilution using the BSA sample diluent (see 4.2). If the absorbance results indicate that the value for the 1:50 dilution is greater than 32 ng/mL, then repeat the ELISA assay with appropriately-diluted samples. An example for making dilutions is the following.
- 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.
 - 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 sample of the test article and a spiked buffer control. Spiked samples can be prepared as follows:
- A spiked test article can be made up directly in the microtiter strip well. For a spiked concentration of 8 ng/mL, do a 4x dilution into the well using the 32 ng/mL Cygnus, Inc. standard. For example, add 12.5 µL of the 32 ng/mL standard directly into the well containing 37.5 µL of the sample test article prepared in step 6.1.1. 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 spiked-buffer control, add 12.5 µL of the 32 ng/mL standard directly into the well containing 37.5 µL of the buffer provided by the requestor. This produces 8 ng/mL concentration of BSA 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.

6.2 Standard Preparation

6.2.1 Prepared standards at 0, 0.5, 2, 8, and 32 ng/mL are included in the Cygnus BSA kit. Because of the possible background levels of BSA, the prepared 2 ng/mL standard is used as the lowest standard point on the curve. In order to increase the number of points on the standard curve, at least one more standard, a 4 ng/mL or a 16 ng/mL standard, is prepared fresh immediately before use. For example:

- To make a 4 ng/mL standard, do a 1:1 dilution with the 8 ng/mL standard. Add 25 μ L of the 8 ng/mL standard to 25 μ L of the diluent directly into the 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.
- To make a 16 ng/mL standard, do a 1:1 dilution with the 32 ng/mL standard. Add 25 μ L of the 32 ng/mL standard to 25 μ L of the diluent directly into the microtiter strip well. This will need to be done in duplicate. Draw 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 4 ng/mL or 16 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 Direct-Q water. Log the wash solution in the PA Solutions logbook. Label the solution bottle with the QCB number, initials, date prepared, and expiration date. Refer to **SOP 22702, Solutions Used in Process Analytics**.

7.0 Procedure

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

7.3 Pipette 100 μ L of **anti-BSA:HRP** into each well.

7.3.1 Pour contents of **anti-BSA: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 into a Ziploc bag (4.7).

7.5 Transfer to the microtiter plate shaker (4.6) and incubate for 1 hour \pm 2 minutes at a setting of "2" (180 rpm).

- 7.6 Using a manual microtiter plate procedure, wash the plate with at least 350 μ L 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 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 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 step, 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 the 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 about 60 seconds to drain. Strike the plate again for about 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 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 substrate.

- 7.7 Pipette 100 μ L of the **TMB 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 μ L of substrate plus 100 μ L of stop solution against a water blank. If the absorbance is greater than 0.1, it may be necessary to obtain new substrate (substrates are not kit specific) or the sensitivity of the assay may be compromised.

- 7.8 Place the microtiter plate into a new Ziploc bag. Incubate at room temperature for 30 minutes \pm 2 minutes.

- 7.9 Remove the plate from the Ziploc bag and pipette 100 μ L of the **stop solution** as in step 7.3.
- 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.

Note: Read the plates within 30 minutes after adding the stop solution since the color will fade over time.

8.0 Data Analysis and Acceptable Criteria

After the Ascent BSA 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 "scidata on [REDACTED]:"
- 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 "BSA 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 > 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% \pm 30 of what is "Expected."
- 8.7 Fill in the "Found" Spike Buffer Control and the "% CV." The spike 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% \pm 30 of what is "Expected."
- 8.8 Fill in replicates 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 test article must exhibit dilution-corrected analyte concentrations that vary no more than $\pm 50\%$ between dilutions. Avoid consideration of dilutional data where the assay value before dilution correction falls below two times the LOQ of the assay (2 ng/mL for the BSA 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 Previous Dilution
Neat	100	N/A
1:5	50	50
1:25	45	90
1:50	47	104

- 8.10 If the criteria in sections 8.8 and 8.9 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 BSA 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 < 2 ng/mL.

- 9.3 Record all solution preparation on Form 22119-01, BSA ELISA Preparation, (Attachment 4) and include with the QC Test Request form.

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 Bovine Serum Albumin 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 Ascent BSA ELISA Results
- 11.3 **Attachment 3** Sample EXCEL Summary Worksheet
- 11.4 **Attachment 4** Form 22119-01, BSA ELISA Preparation
- 11.5 **Attachment 5** Cygnus Technologies Inc. BSA ELISA Kit Insert

Attachment 1 Sample ELISA Work List

H	G	F	E	D	C	B	A	↑
EMPTY	32 ng/ml Standard	16 ng/ml Standard	8 ng/ml Standard	4 ng/ml Standard	2 ng/ml Standard	0 ng/ml Standard	BLANK 0 ng/ml Std	1
EMPTY	32 ng/ml Standard	16 ng/ml Standard	8 ng/ml Standard	4 ng/ml Standard	2 ng/ml Standard	0 ng/ml Standard	BLANK 0 ng/ml Std	2
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED Buffer CONTROL	Buffer CONTROL	3
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED Buffer CONTROL	Buffer CONTROL	4
								5
								6
								7
								8
								9
								10
								11
								12

Assay:

Sample ID:

Date:

Analyst:

Attachment 2 Sample Ascent BSA ELISA Results

Ascent Software

Curve Fit1

11/25/03 11:24 AM

Session: [REDACTED]
Instrument: EMS Reader MF V2.9-0D
User name: [REDACTED]
Started at: 10/30/03 0:32:8PM
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	_014	_014				
B	Cal1	Cal1	snc	snc	s014	s014				
C	Cal2	Cal2	buf	buf						
D	Cal3	Cal3	sbuf	sbuf						
E	Cal4	Cal4	_014	_014						
F	Cal5	Cal5	s014	s014						
G	Cal6	Cal6	_014	_014						
H			s014	s014						

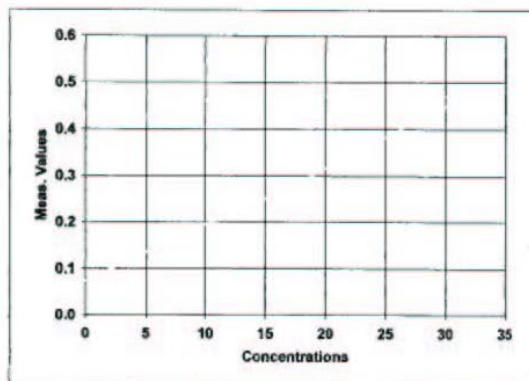
Source data for calibrators Sheet: Measure1, Assay: Assay1 and for samples Sheet: Measure1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	0.071	0.071	0.069	0.068	1.891	0.515				
B	0.074	0.072	0.138	0.134	0.502	0.475				
C	0.095	0.099	0.081	0.077						
D	0.130	0.114	0.132	0.121						
E	0.174	0.169	0.185	0.176						
F	0.261	0.262	0.239	0.229						
G	0.534	0.420	0.207	0.198						
H			0.288	0.289						

Sheet: Measure1, Assay: Assay1

Name	Meas.	Conc.
Cal1	0.074 0.072 0.073	0.00
Cal2	0.095 0.099 0.097	2.00
Cal3	0.130 0.114 0.122	4.00
Cal4	0.174 0.169 0.172	8.00
Cal5	0.261	

	Conc.	Meas.	CalcConc.	Residual
Cal1	0	0.073	0	0
Cal2	2	0.097	2	0
Cal3	4	0.122	4	0
Cal4	8	0.172	8	0
Cal5	16	0.262	16	0
Cal6	32	0.477	32	0



Attachment 3 Sample EXCEL Summary Worksheet

Summary of BSA ELISA Results

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

Sample ID: [REDACTED] Project Name: [REDACTED] Lot #: LXX00000

Kit Lot#: [REDACTED] Expiration Date: 1/31/2011

BSA 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 "BSA Spiking Solution" (ng/mL): 32.0

Correlation Coefficient of Standard Curve: point to point

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

Avg. Positive Control (ng/mL):	Expected	Found	%Recovery	%CV
	4.00	3.95	98.75%	7.88
Spiked Buffer Control (ng/mL)	8.00	7.52	94.00%	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)

Spiked Test Article Concentrations (ng/mL; not corrected for dilution):					
Acceptable Criteria: %CV <25%					
	<u>Dilution</u> <u>Factor</u>	<u>Replicate 1</u>	<u>Replicate 2</u>	<u>Average</u>	<u>%CV</u>
	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%					
	<u>Dilution</u> <u>Factor</u>	<u>Replicate 1</u>	<u>Replicate 2</u>	<u>Average</u>	<u>%CV</u>
	1	1287.70	1276.00	1281.85	0.65
	5	1112.60	1032.00	1072.30	5.32
	10	807.10	918.00	862.55	9.09
	20	1034.40	999.10	1016.75	2.45
	40	1044.65	979.65	1012.15	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

NCI-Frederick
Form No.: 22119-01
SOP No.: 22119
Revision 03:

Page 1 of 2

BSA 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: _____ μ L

Volume of test sample: _____ μ L

Second dilution: _____

Volume of diluent: _____ μ L

Volume of initial diluted test sample _____ μ L

Fourth dilution: _____

Volume of diluent: _____ μ L

Volume of third diluted test sample _____ μ L

Third Dilution: _____

Volume of Diluent: _____ μ L

Volume of Second Diluted sample _____ μ L

Fifth dilution: _____

Volume of diluent: _____ μ L

Volume of fourth diluted test sample _____ μ L

Test sample #2

Name: _____ Appearance: _____

Lot # _____

Protein Concentration: _____ mg/mL

Initial dilution: _____

Volume of diluent: _____ μ L

Volume of test sample: _____ μ L

Second dilution: _____

Volume of diluent: _____ μ L

Volume of initial diluted test sample _____ μ L

Fourth dilution: _____

Volume of diluent: _____ μ L

Volume of third diluted test sample _____ μ L

Third Dilution: _____

Volume of Diluent: _____ μ L

Volume of Second Diluted sample _____ μ L

Fifth dilution: _____

Volume of diluent: _____ μ L

Volume of fourth diluted test sample _____ μ L

Test sample #3

Name: _____ Appearance: _____

Lot # _____

Protein Concentration: _____ mg/mL

Initial dilution: _____

Volume of diluent: _____ μ L

Volume of test sample: _____ μ L

Attachment 4 (Continued)

NCI-Frederick
Form No.: 22123-01
SOP No.: 22123
Revision 03:

Page 2 of 2

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

Fourth dilution : _____
Volume of diluent: _____ μ L
Volume of third diluted test sample _____ μ L

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

Fifth Dilution _____
Volume of Diluent: _____ μ L
Volume of fourth diluted test sample _____ μ L

Positive Control

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

Spiked Buffer Control

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

Template Procedure**anti-mouse immunoglobulin:alkaline phosphatase**

Lot# _____
Expiration Date: _____
Volume of antibody added to wells: _____
Incubation Time: _____
Washing times: _____

Substrate

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

Data Storage

File Name: _____

Performed by/Date: _____

Reviewed by/Date: _____

Attachment 5 Cygnus Technologies Inc. BSA ELISA Kit Insert



Bovine Serum Albumin (BSA) Assay

Immunoenzymetric Assay for the Measurement of BSA Catalog # F030

Intended Use

This kit is intended for use in quantitating bovine serum albumin (BSA). The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

The manufacture of products by various biotechnological processes such as cell or tissue culture can result in residual contamination of the desired product by components used in the culture media. The use of so called serum free defined media greatly reduces the number of potential contaminants but it may still be necessary to determine trace contamination levels of the proteins and growth factors used in these media. Most commercial formulations of serum free media contain significant amounts of albumin and transferrin either of bovine or human origin, and insulin from various species. When the intended product may be used as a therapeutic agent in humans or animals the product should be highly purified to avoid potential health risks or other problems that might result from trace contaminants. Efforts to reduce trace media contamination to the lowest levels practical through optimal process design, validation, and final product testing require a highly sensitive and reliable analytical method. This BSA ELISA assay is designed to provide a simple to use, precise, and highly sensitive method to detect BSA contamination to less than 250 pg/mL. As such, this kit can be used as a tool to aid in optimal purification process development and in routine quality control of in-process streams as well as final product.

Principle of the Procedure

This BSA assay is a two-site immunoenzymetric assay. Samples containing BSA are reacted in microtiter strips coated with an affinity purified capture antibody. A second anti-BSA antibody labeled with the enzyme horseradish peroxidase (HRP) is reacted simultaneously forming a sandwich complex of solid phase antibody-BSA-HRP labeled antibody. After a wash step to remove any unbound reactants the strips are then reacted with Tetramethyl benzidine (TMB) substrate. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of BSA present. Accurate quantitation is achieved by comparing the signal of unknowns to BSA standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-BSA:HRP Sheep polyclonal antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F033
Anti-BSA coated microtiter strips 12x8 well strips in a bag with desiccant	F032*
BSA Standards BSA in a protein matrix with preservative. Standards at 0, 0.5, 2, 8, and 32ng/mL. 1mL/vial	F031
Stop Solution 0.5N 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 # F032.

Storage & Stability

* All reagents should be stored at 2°C to 8°C for stability until the expiration date printed.

* The substrate reagent should not be used if its stopped absorbance at 450nm is greater than 0.1.

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

Materials & Equipment Required But Not Provided

Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm. (If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)
Pipettors - 50µL and 100µL
Repeating or multichannel pipettor - 100µL
Microtiter plate rotator (150 - 200 rpm)
Sample Diluent (recommended Cat # F031A)
Distilled water
1 liter wash bottle for diluted wash solution

Attachment 5 (Continued)

Precautions

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

Preparation of Reagents

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

Procedural Notes

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 0ng/mL standard is greater than 0.200, evaluate plate washing procedure for proper performance.
2. This kit is a very sensitive assay for BSA (< 250pg/mL). Since BSA is a common reagent in many laboratories and is often used at relatively high concentrations of more than a million fold higher, it is very important to use extreme care to avoid contamination of any of the reagents in this kit with external sources of BSA. BSA contamination will manifest itself as either high assay background, poor precision, or unexpected results.
3. Dilution of samples will be required for samples >32ng/mL. The diluent used should be compatible with accurate recovery. The preferred diluent is our Cat. # F031A 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 F031A its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents should be validated in the assay to demonstrate that they do not give elevated background and are not contaminated with BSA. The diluent should also give acceptable recovery when spiked with known quantities of BSA.
4. High Dose Hook Effect may be observed in samples with very high concentrations of BSA. Samples greater than 10µg/mL may give absorbances less than the 32ng/mL standard. If a hook effect is possible, samples should also be assayed diluted. If the dilution corrected concentration of the diluted sample is greater than the undiluted samples this may be indicative of the hook effect.

Limitations

- * Cross reactivity of these antibodies with albumin from other species has not been extensively investigated. No interference from human, sheep, or rabbit albumin has been demonstrated at a 1mg/mL concentration.
- * Certain sample matrices may interfere in this assay. Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration, high salt concentration, extremes of pH (<6.0 and >8.5) or very high protein concentrations may give erroneous results. It is recommended to test the sample matrix for interference by diluting the 32ng/mL standard 1 part to 3 parts of the matrix which does not contain any BSA. This diluted standard when assayed as an unknown should give a value of 6 to 10ng/mL. In cases where BSA levels in the sample will allow for sample dilution, such dilution will often overcome sample matrix interference. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices

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 for the immunological step. These can be purchased from most laboratory supply companies. Alternatively you can purchase an approved, pre-calibrated shaker directly from *Cygnus Technologies*. 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 30 minutes 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.**
- * 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 BSA levels in that sample.
- * 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 wavelength. Blank the instrument using the zero standard wells after assay completion.
- * All standards, controls and samples should be assayed in duplicate. Samples that could contain very high levels of BSA above the 32ng/mL standard or in the "Hook" region of this assay should also be assayed diluted. Recommended diluent is *Cygnus Technologies* Cat # F031A. Avoid the use of diluents

Attachment 5 (Continued)

which contain NaN_3 or could be contaminated with trace levels of BSA.

* 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.

* 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.

* 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 $100\mu\text{L}$ of substrate plus $100\mu\text{L}$ of Stop Solution against a water blank. If the absorbance is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

* Plates should be read within 30 min. after adding stop since color will fade over time.

Assay Protocol

1. Pipette $50\mu\text{L}$ of standards, controls and samples into wells indicated on work list.
2. Pipette $100\mu\text{L}$ of anti-BSA:HRP (#F033) into each well.
3. Cover & incubate on rotator at $\sim 180\text{rpm}$ for 1 hour at room temperature, $24^\circ\text{C} \pm 4^\circ$.
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 pipetting in $\sim 350\mu\text{L}$. 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 $100\mu\text{L}$ of TMB substrate (#F005).
6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.
7. Pipette $100\mu\text{L}$ of Stop Solution (#F006).
8. Read absorbance at 450/650nm blanking on the Zero standard.

Quality Control

* Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 1ng/mL . CVs for samples $<1\text{ng/mL}$ may be greater than 10%.

* For optimal performance the absorbance of substrate when blanked against water should be <0.1 .

* It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

Example Data

Well #	Contents	Abs. At 450nm	Mean Abs.	ng/mL BSA
1A	Zero Std	0.000		
1B	Zero Std	0.002	0.001	
1C	0.5ng/mL	0.035		
1D	0.5ng/mL	0.031	0.033	
1E	2ng/mL	0.127		
1F	2ng/mL	0.115	0.121	
1G	8ng/mL	0.385		
1H	8ng/mL	0.371	0.377	
2A	32ng/mL	1.312		
2B	32ng/mL	1.328	1.319	
2C	sample 1	0.005		
2D	sample 1	0.010	0.008	$<0.2\text{ng/mL}$
2E	sample 2	0.127		
2F	sample 2	0.123	0.122	2ng/mL

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL. This data reduction may be performed through computer methods using curve-fitting routines such as point-to-point, 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.

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. A more detailed copy of this "Validation Summary" report can be obtained by request. This validation is generic in nature and is intended to supplement but not replace certain user and sample specific qualification and validation that should be performed by each laboratory. At a minimum each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your samples types containing BSA 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. Each laboratory and technician should also demonstrate competency in the assay by performing a precision

Attachment 5 (Continued)

study similar to that described below. A more detailed discussion of recommended user validation protocols can be obtained by contacting our Technical Services Dept. or on-line at our web site.

Sensitivity

The lower limit of detection (LOD) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. LOD is ~125 pg/mL in the recommended protocol. The lower limit of quantitation (LOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are <20%. The LOQ is ~250 pg/mL.

Precision

Precision is defined as the percent coefficient of variation (%CV). This is calculated by dividing the standard deviation by the mean value for a number of replicate determinations of two different control samples in the low and high concentration range of the assay. Both intra and inter-assay (n=5 assays) precision were determined on 2 pools with low (~2ng/mL) and high concentrations (~12ng/mL).

Intra-assay			Inter-assay		
# of tests	Mean ng/mL	%CV	# of assays	Mean ng/mL	%CV
12	2.0	7.4	5	2.1	8.1
20	12.2	5.3	5	12.2	6.7

Specificity/Cross-Reactivity

In sandwich ELISA cross reactivity can manifest itself either as a false increase in BSA (positive cross reactivity) or as a false decrease in BSA (negative cross reactivity) when BSA present in the sample competes with the cross reactant for the kit antibodies. The following materials were tested for cross reactivity at the concentrations indicated both in the absence of BSA and in the presence of 15 ng/mL BSA. None of these materials were found to yield any statistically significant false increase or decrease in apparent BSA concentrations. While no cross reactivity was detected in any of the substances tested, it is recommended that each user test known materials in their sample matrices for cross reactivity in a similar experiment.

Materials Not Cross Reactive for BSA	
Substance	Concentration Tested
Human albumin	10 mg/mL
Goat serum	10%
Mouse serum	10%
Rabbit serum	10%
Porcine gelatin	10mg/mL
Fish gelatin	1%

Recovery/ Interference Studies

Various buffer matrices were evaluated by adding known amounts of BSA. 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 (<5.0 and >8.5), high salt concentrations, as well as certain detergents can cause under-recovery. Some product proteins in high concentration may also interfere in the accurate measurement of BSA. Each user should validate that their sample matrices yield accurate recovery. Such an experiment can be easily performed by diluting the 32ng/mL standard provided with this kit into the sample matrix in question. For example, add 1 part of the 32ng/mL standard to 3 parts of the matrix containing no or very low BSA contaminants. This diluted standard when assayed as an unknown should give a value of 6 to 10 ng/mL. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Hook Capacity

Increasing concentrations of BSA > 32 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 32 ng/mL standard, was 10 µg/mL.

Ordering Information/ Customer Service

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

www.cygnustechnologies.com
Cygnus Technologies, Inc.
4701 Southport Supply Rd. SE, Suite 7
Southport, NC 28461 USA
Tel: 910-454-9442
Fax: 910-454-9443
Email: techsupport@cygnustechnologies.com