Title: Quantitation of Protein A Using the Cyngus Inc. ELISA Kit

SOP Number: 22189

Revision Number: 03

Supersedes: Revision 02

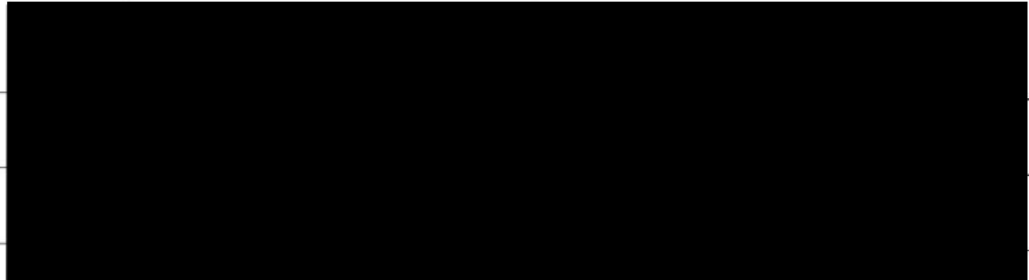Effective Date : NOV 07 2017

Originato r/Date: _____

Approval/Date: _____

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Table of Contents

1.0   Purpose

This SOP describes the use of the Cygnus, Inc. ELISA kit for residual Protein A quantitation.

2.0   Scope

This SOP applies to PA/QC personnel who will perform the Protein A ELISA.

3.0   Authority and Responsibility

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

3.2   Process Analytics/Quality Control (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   Equipment, Supplies and Reagents

4.1   Mix-N-Go Protein A ELISA kit, BDP PN 31126, Cygnus Technologies, Catalog Number F600 for natural and recombinant Protein A.

OR

Mix-N-Go Protein A ELISA kit, BDP PN 31127, Cygnus Technologies, Catalog Number F610 for unnatural Protein A constructs such as MabSelect SuRe™.

4.2   Single-channel and Multi-channel pipettors.

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

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

4.5   Optical Adhesive Covers, BDP PN 21142 or BDP approved equivalent.

4.6   Ziploc Bag, BDP PN 20339, or BDP approved equivalent.

4.7   Reagent reservoirs, BDP PN 20270, BDP PN 20481 or BDP approved equivalent.

4.8   Microcentrifuge tubes 0.5 mL, BDP PN 21369, or BDP approved equivalent.

4.9   Type I (Ultrapure) water or equivalent.

4.10   Liter Graduated Cylinder for wash solution.

4.11   Squirt/wash bottle with the tip cut off.

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

4.13   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 Protein A Standards, ejecting pipette tips, etc. Pipette tips possessing an aerosol barrier must be used.

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

5.5   Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration, extremes of pH (< 6.0 and > 8.5), very high buffer molarity, 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 kit and reagents 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. The required minimum sample volume for this assay is 1 mL.

6.1   Sample Preparation: Linear Range of Assay = 0 – 10 ng/mL

   6.1.1   Prepare the test article immediately before use undiluted and at recommended 1:5 and 1:50 dilutions using the Sample Diluent (Cat# I600). If the absorbance results indicate that the value for the 1:50 dilution is greater than 10 ng/mL, then repeat the assay with appropriately diluted samples. (The original data is attached to the QC test request.) An example for making dilutions is the following:

   - For duplicate measurements of spiked and unspiked diluted test article (a total of four 100 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 100 µL of the 1:5 diluted test article to 900 µL diluent.

   6.1.2   Prepare a spiked sample of the test article. For a spiked Protein A concentration of 2.5 ng/mL, mix 1 part of the 10 ng/mL standard with 3 parts of sample. For example, add 50 µL of the 10 ng/mL standard to 150 µL of the sample. Vortex to mix.

6.2   Standards (STD #F603 – Kit# F600, STD #F613 – Kit# F610)

   6.2.1   Prepared standards at 0, 0.16, 0.31, 0.63, 1.25, 2.5, 5 and 10 ng/mL are included in the ELISA kit.

6.3   Positive, Negative, and Spike Control Preparation

   6.3.1   The 2.5 ng/mL standard serves as the positive control.

   6.3.2   The negative control is the Sample Diluent (Cat# I600).

   6.3.3   For a spiked Protein A concentration of 2.5 ng/mL prepare a 4x dilution using the 10 ng/mL standard. For example, add 50 µL of the 10 ng/mL standard to 150 µL of the Sample Diluent (Cat# I600). Vortex to mix.

6.4   Wash Solution Preparation

   6.4.1   Empty the entire contents of the wash solution bottle, 50 mL, into a 1 L graduated cylinder. Bring to 1 L with Ultrapure water. Log the wash solution in the PA/QC Solutions Logbook. Label the solution bottle with the PA number, initials, date prepared, and expiration date. Refer to *SOP 22702 – Solutions used in Process Analytics*.

7.0   Procedure

7.1   Sample Treatment - Denaturation

   7.1.1   Transfer 100 µL of standards, prepared controls and samples to the Sample Treatment Plate (STP).

7.1.2   Add 50 µL of Mix-N-Go Denaturing Buffer (#F604) to each well. Mix by pipetting up and down 10 - 20 times.

7.1.3   Incubate at room temperature for 5 - 10 minutes.

7.2   Prepare an ELISA Worklist (**Attachment 1**) by labeling the wells where the samples and standards will be placed. Count the microtiter strips needed. Remove the required number of microtiter strips from the kit and place them in the provided frame.

7.3   Pipette 100 µL of the anti-Protein A:HRP detection antibody (#F601) into each well of the antibody coated microtiter plate (#F052).

7.4   Pipette 25 µL of denatured standards, controls and samples into wells indicated on the ELISA Worklist (**Attachment 1**). All standards, controls and samples must be assayed in at least duplicate.

7.5   Cover plate with an Optical Adhesive Cover and place the plate in a Ziploc bag. Incubate on a microtiter plate shaker set to 500 rpm for 1 hour at room temperature.

7.6   Perform Manual Plate Wash. Flip contents from plate to waste and wash 4 times using a squirt bottle or by pipetting. Fill all wells to capacity with each wash. Blot the plate inverted on absorbent paper after each wash.

7.7   Pipette 100 µL of TMB substrate (#F005) into each well. Incubate at room temperature for 30 minutes. Do not shake.

7.8   Pipette 100 µL of Stop Solution (#F006) into each well.

7.9   Read the absorbance at 450/620 nm. Refer to ***SOP 22100 - Operation of the Labsystems iEMS Microtiter Plate Reader/ Dispenser*** for analysis of the data.

8.0   Data Analysis and Acceptable Criteria

The Ascent Protein A Results (**Attachment 2**) will need to be manually entered into the Summary Worksheet (**Attachment 3**). The Summary Worksheet is in Microsoft Excel format. The transcribed data from the Ascent report is calculated by the Excel application. The acceptable spike recoveries, dilutional linearity and %CV's are calculated in this Summary Worksheet. Outlier testing may be performed using standard statistical methods. If >1 point is identified as an outlier, the test is invalid.

8.1   Select the ▮▮▮▮▮▮▮▮▮▮▮▮▮▮ folder. Open the Mix-N-Go Protein A Template.

8.2   The raw absorbance value for the 10 ng/mL standard must be > 0.7 au. If the 10 ng/mL standard does not meet the criteria, then the assay is invalid and will need to be rerun. Enter the raw absorbance at 450 nm in the corresponding cell. The spreadsheet will display 'PASS' if the value entered is greater than 0.7 or 'Invalid Test' if the value is ≤ 0.7.

8.3   Fill in the top portion of the summary sheet: QC number, Analyst, Date, Sample ID, Lot Number, Kit Catalogue Number (F600 or F610), Lot Number and Expiration Date.

8.4   Fill in the "Protein A Concentration" section with appropriate measured concentrations 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 - the Ascent software does not truncate intermediate values during calculations. The "%CV" should be less than 25% for samples > 0.31 ng/mL.

8.5   Fill in the "Expected" and "Found" Positive Control and the "%CV" from the Ascent ELISA Results. The "%CV" should be less than 25%. The "Found" positive control should be 100 $\pm$ 30% of what is "Expected".

8.6   Fill in the "Found" Negative Control from the Ascent ELISA Result. This number should be close to 0 or less than minimum (< min).

8.7   Fill in the "Found" Spike Control and the "%CV." 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 test article must exhibit dilution-corrected analyte concentrations that vary no more than $\pm$ 50% between dilutions. Avoid consideration of the dilutional data where the assay value before dilution correction falls below the lowest standard (0.16 ng/mL) for the PROTEIN A assay). The first dilution-corrected concentration exhibiting dilutional linearity is reported. In the following example 8.2 ng/mL would be the reported result.

   Example:

      Undilute sample: 8.2 ng/mL N/A

      1:5 dilution: 6.5 ng/mL   79.3% of previous

      1:50 dilution: 2.3 ng/mL   35.4% of previous

8.10 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%.

8.11 If %CV and recoveries for the assay controls do not meet these criteria, then the assay is invalid and needs to be rerun.

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 Protein A 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 < 0.16 ng/mL.

9.3   Record all solution preparation on Form 22189-01 Protein A ELISA Preparation Form (**Attachment 4**) and include with the QC Test Request form.

10.0 References

10.1 Cygnus Technologies Mix-N-Go Protein A ELISA Kit Instructions (**Attachment 5**).

10.2 Cygnus Suggested Manual Microtiter Plate Washing Procedure.

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

10.4 **SOP 22702**   *Solutions used in Process Analytics*

11.0 Attachments

    11.1 Attachment 1   Sample ELISA Worklist

    11.2 Attachment 2   Example Ascent Protein A ELISA Result

    11.3 Attachment 3   Sample EXCEL Summary Worksheet

    11.4 Attachment 4   **Form 22189-01**, Protein A ELISA Preparation Form

    11.5 Attachment 5   Cygnus Technologies Mix-N-Go Protein A Assays

    11.6 Attachment 6   Cygnus Technologies Manual Plate Washing

ATTACHMENT 1

Sample ELISA Worklist

Mix-N-Go Protein A ELISA Worklist

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 ng/mL	0 ng/mL		Sample 1 Undilute	Sample 1 Undilute		Spiked Sample 1 Undilute	Spiked Sample 1 Undilute				
B	0.16 ng/mL	0.16 ng/mL		Sample 1 1:5	Sample 1 1:5		Spiked Sample 1 1:5	Spiked Sample 1 1:5				
C	0.31 ng/mL	0.31 ng/mL		Sample 1 1:50	Sample 1 1:50		Spiked Sample 1 1:50	Spiked Sample 1 1:50				
D	0.63 ng/mL	0.63 ng/mL		Sample 2 Undilute	Sample 2 Undilute		Spiked Sample 2 Undilute	Spiked Sample 2 Undilute				
E	1.25 ng/mL	1.25 ng/mL		Sample 2 1:5	Sample 2 1:5		Spiked Sample 2 1:5	Spiked Sample 2 1:5				
F	2.5 ng/mL	2.5 ng/mL		Sample 2 1:50	Sample 2 1:50		Spiked Sample 2 1:50	Spiked Sample 2 1:50				
G	5 ng/mL	5 ng/mL		Buffer	Buffer		Spiked Buffer	Spiked Buffer				
H	10 ng/mL	10 ng/mL										

ATTACHMENT 2

Example Ascent Protein A ELISA Result

Session: C:\ASCENT\Mix-N-GoProtA.SEE
Instrument: EMS Reader MF V2.9-0D
User name: Dale Ruby
Started at: 9/27/2017 2:54:29PM
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	11	12
A	Cal1	Cal1		S1 Un	S1 Un		SPK S1 Un	SPK S1 Un				
B	Cal2	Cal2		S1 1:5	S1 1:5		SPK S1 1:5	SPK S1 1:5				
C	Cal3	Cal3		S1 1:50	S1 1:50		SPK S1 1:50	SPK S1 1:50				
D	Cal4	Cal4		S2 Un	S2 Un		SPK S2 Un	SPK S2 Un				
E	Cal5	Cal5		S2 1:5	S2 1:5		SPK S2 1:5	SPK S2 1:5				
F	Cal6	Cal6		S2 1:50	S2 1:50		SPK S2 1:50	SPK S2 1:50				
G	Cal7	Cal7		Buffer	Buffer		Buffer	Buffer				
H	Cal8	Cal8										

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	11	12
A	0.029	0.027		0.600	0.550		1.250	1.213				
B	0.080	0.077		0.060	0.062		0.845	0.847				
C	0.138	0.135		0.006	0.007		0.805	0.805				
D	0.257	0.264		0.600	0.550		1.250	1.213				
E	0.488	0.491		0.060	0.062		0.845	0.847				
F	0.880	0.917		0.006	0.007		0.805	0.805				
G	1.800	1.780		0.022	0.022		0.890	0.910				
H	3.017	3.083										

Sheet: Precalc1, Assay: Assay1

Name	Meas.	Conc. (ng/mL)
Cal1	0.029	
	0.027	
	0.028	0
Cal2	0.080	
	0.077	
	0.079	0.16
Cal3	0.138	
	0.135	
	0.137	0.31
Cal4	0.257	
	0.264	
	0.261	0.63
Cal5	0.488	
	0.491	
	0.490	1.25
Cal6	0.880	
	0.917	
	0.899	2.5
Cal7	1.800	
	1.780	
	1.790	5
Cal8	3.017	
	3.083	
	3.050	10

	Conc.	Meas.	CalcConc.	Residual
Cal1	0	0.028	0	0
Cal2	0.16	0.079	0.16	0
Cal3	0.31	0.137	0.31	0
Cal4	0.63	0.261	0.63	0
Cal5	1.25	0.490	1.25	0
Cal6	2.5	0.899	2.5	0
Cal7	5	1.790	5	0
Cal8	10	3.050	10	0
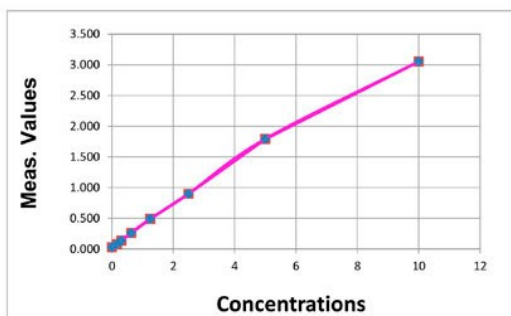
Status:
Fit type: Point to point
Meas. transformation: Linear
Conc. transformation: Linear

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ATTACHMENT 2 (Continued)

Example Ascent Protein A ELISA Result

Calculated concentrations Sheet: Precalc1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.16379	-0.25822		1.696	1.533		3.812	3.690				
B	0.002279	-0.00749		-0.063	-0.056		2.493	2.498				
C	0.191143	0.181374		-0.239	-0.235		2.361	2.364				
D	0.578639	0.601433		1.696	1.533		3.812	3.690				
E	1.330837	1.340606		-0.063	-0.056		2.493	2.498				
F	2.607294	2.727776		-0.239	-0.235		2.361	2.364				
G	5.603061	5.537936		-0.187	-0.187		2.640	2.705				
H	9.565939	FALSE										

Calibrators and samples Sheet: Precalc1, Assay: Assay1

Name	Meas.	Calc.	Dil. factor	Result	SD	CV-%
Cal1	0.029	-0.16	1	-0.16		
	0.027	-0.17	1	-0.17		
	0.028	0.00		-0.18	0.005	-2.60
Cal2	0.080	0.00	1	0.00		
	0.077	-0.01	1	-0.01		
	0.079	0.16		-0.01	0.007	-54.80
Cal3	0.138	0.19	1	0.19		
	0.135	0.18	1	0.18		
	0.137	0.31		0.18	0.007	3.92
Cal4	0.257	0.58	1	0.58		
	0.264	0.60	1	0.60		
	0.261	0.63		0.58	0.016	2.78
Cal5	0.488	1.33	1	1.33		
	0.491	1.34	1	1.34		
	0.490	1.25		1.33	0.007	0.52
Cal6	0.880	2.61	1	2.61		
	0.917	2.73	1	2.73		
	0.899	2.50		2.66	0.085	3.21
Cal7	1.800	5.60	1	5.60		
	1.780	5.54	1	5.54		
	1.790	5.00		5.56	0.046	0.83
Cal8	3.017	9.57	1	9.57		
	3.083	9.78	1	9.78		
	3.050	10.00		9.66	0.152	1.57
Sam1	0.600	1.70	1	1.70		
	0.550	1.53	1	1.53		
	0.575	-0.01		1.60	0.115	7.18
	0.060	-0.06	5	-0.31		
	0.062	-0.06	5	-0.28		
	0.061	-0.01		-0.31	0.023	-7.48
	0.006	-0.24	50	-11.93		
	0.007	-0.24	50	-11.77		
	0.007	0		-11.86	0.115	-0.97
Sam2	0.600	1.70	1	1.70		
	0.550	1.53	1	1.53		
	0.575	0.02		1.60	0.115	7.18
	0.060	-0.06	5	-0.31		
	0.062	-0.06	5	-0.28		
	0.061	0.02		-0.31	0.023	-7.48
	0.006	-0.24	50	-11.93		
	0.007	-0.24	50	-11.77		
	0.007	0.02		-11.86	0.115	-0.97

ATTACHMENT 2 (Continued)

Example Ascent Protein A ELISA Result

BUFFER	0.02	-0.19	1	-0.19		
	0.02	-0.19	1	-0.19		
	0.02	-0.19		-0.20	0.000	0.00
Spike	1.25	3.81	1	3.81		
Sample1	1.21	3.69	1	3.69		
	1.23	3.75		3.74	0.086	2.31
	0.85	2.49	5	12.47		
	0.85	2.50	5	12.49		
	0.85	2.50		12.47	0.017	0.14
	0.80	2.36	50	118.07		
	0.81	2.36	50	118.19		
	0.80	2.36		118.12	0.086	0.07
Spike	1.25	3.81	1	3.81		
Sample2	1.21	3.69	1	3.69		
	1.23	3.75		3.74	0.086	2.31
	0.85	2.49	5	12.47		
	0.85	2.50	5	12.49		
	0.85	2.50		12.47	0.017	0.14
	0.80	2.36	50	118.07		
	0.81	2.36	50	118.19		
	0.80	2.36		118.12	0.086	0.07
SBUFFER	0.89	2.64	1	2.64		
	0.91	2.70	1	2.70		
	0.90	2.67		2.66	0.046	1.73

ATTACHMENT 3

Sample EXCEL Summary Worksheet

Summary of Protein A (Mix-N-Go) ELISA Results

QC Number:	QC-000000	**Analyst:**	Dale Ruby	**Date:**	9/26/2017
Sample ID:		Sample Name		**Lot #:**	L0000000
Kit Lot#:	18612	**Expiration Date:**	9/27/2017	**Kit Cat#** F600	

Protein A Concentration (ng/ml)
Acceptable Criteria: %CV <25% for samples > 0.31 ng/ml

Dilution Factor	Measured Concentration		Corrected Concentration		Average	%CV
	Replicate 1	Replicate 2	Replicate 1	Replicate 2		
1.00	1.70	1.53	1.70	1.53	**1.62**	7.2
5.00	0.00	0.00	0.00	0.00	0.00	N/A
50.00	0.00	0.00	0.00	0.00	0.00	N/A

Final Result: **1.62 ng/mL**

Corrected Concentration = Measured Concentration x dilution factor

*n/c = not calculated

Calculated Concentration of "Protein A Spiking Solution" (ng/ml):   10.0

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):	2.50	2.66	106%	3.21
Spiked Buffer Control (ng/mL)	2.50	2.66	106%	1.73

Dilutional Linearity

Dilution Factor		Average Dilution Corrected	Percent Change in Dilution Corrected Concentration from Previous Value
1.00		**1.62**	**N/A**
5.00		0.00	0.0
50.00		0.00	#DIV/0!

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

Raw Mean Absorbance of 10 ng/mL Standard (> 0.7 au):   3.1 au   **PASS**

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	3.81	3.69	**3.75**	**2.31**
5	2.49	2.50	2.50	0.14
50	2.36	2.36	2.36	0.07

Percent Spike Recovery of Test Article:

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

Dilution Factor	Replicate 1	Replicate 2	Average	%CV
1	100.93	101.17	**101.05**	**0.17**
5	99.60	100.00	99.80	0.28
50	94.40	94.40	94.40	0.00

Calculated by: Spiked test article concentration, ng/ml/[[(0.0118 ml) * (measured concentration, ng/ml) + (0.0062ml)* (Concentration of "Protein A Spiking Solution", ng/ml)] / 0.025 ml]

Analyst/Date: _____

Reviewed By/Date: _____

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ATTACHMENT 4

Form 22189-01, Protein A ELISA Preparation Form

NCI-Frederick
Form No.: 22189-01
SOP No.: 22189
Revision 02: NOV 7 2017

Mix-N-GO PROTEIN A ELISA PREPARATION FORM

Page **1** of **2**

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

Plate Reader MEF Number:_____ Calibration Due Date:_____

Diluent (#I600): Lot#_____ Exp. Date: _____

Test sample Dilutions (Prior To Denaturation):

Test Sample #1
Name:_____
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 Diluent sample _____ µL

Test Sample #3
Name:_____
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 Diluent sample _____ µL

Test Sample #2
Name:_____
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 Diluent sample _____ µL

Test Sample #4
Name:_____
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 Diluent sample _____ µL

Sample Treatment Procedure - Denaturation:
Volume of Samples (100 µL) added to wells of Sample Treatment Plate (#F402): _____µL
Denaturing Buffer (#F604) Lot#:_____ Exp. Date:_____
Volume of Denaturing Buffer (50 µL):_____ µL
Incubation Time (5-10 min at room temperature): _____ minutes

ATTACHMENT 4 (Continued)

Form 22189-01, Protein A ELISA Preparation Form

NCI-Frederick
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Mix-N-GO PROTEIN A ELISA PREPARATION FORM

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Positive Control (2.5 ng/mL, Standard)
Expiration Date:_____
Concentration: _____ ng/mL
Std: _____µL

Negative Control Spike
Preparation of Negative Control Spike
Expiration Date:_____
Concentration: _____ ng/mL
Std: _____µL
Sample Dilution Buffer:_____µL

anti-Protein A:HRP Detection Antibody (#F601)
Lot# _____
Exp. Date:_____
Volume of antibody added to wells:_____ µL
Incubation Time:_____

Microtiter plate shaker: Make: _____ Model: _____

Washing: Number of Washes _____ Volume added to wells (300-400µL) _____µL

TMB Substrate (#F005)
Lot #_____
Expiration Date:_____
Volume of substrate added to wells:_____
Incubation Time:_____

Stop Solution (#F006)
Lot #_____
Expiration Date:_____
Volume of Stop Solution added to wells:_____

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

Data Storage

File Name:_____

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

ATTACHMENT 5

CYGNUS TECHNOLOGIES

Mix-N-Go Protein A Assays
Immunoenzymetric Assays for the Measurement of Protein A

Catalog # F600
and
Catalog # F610

Intended Use

These kits are intended for use in quantitating natural recombinant Protein A (F600) and unnatural Protein A constructs such as MabSelect SuRe™ (F610). These kits are for **Research and Manufacturing Use Only** and are not intended for diagnostic use in humans or animals. The F600 and F610 kits incorporate a well validated sample treatment method to dissociate Protein A from IgG Drug Products **without the need for boiling samples and the associated centrifugation step.** These improvements increase the robustness of the method to accommodate previously problematic immunoglobulins. In doing so, the procedure has been simplified with over 1 hour eliminated from assay processing time.

Summary and Explanation

Protein A, immobilized on various chromatography media, is commonly used to purify antibodies. Even when covalently attached, Protein A can leach off of the chromatography support and co-elute with the antibody. For applications such as the therapeutic use of the antibody, contamination with Protein A must be minimized to avoid any adverse patient effects. Our immunoassay methods provide sensitivity to detect Protein A contamination to less than 100pg/mL. Leached Protein A is typically bound to the product immunoglobulin present in the sample through its Fc region. This binding of Protein A to the product antibody can interfere in the accurate quantitation of Protein A in some immunoassays by inhibiting the ability of the anti-Protein A antibodies used in the assay to bind to the complexed Protein A. This inhibition can result in a significant underestimation of Protein A contamination. Such interference is highly variable from one product antibody to the next.

There are several manufacturers of Protein A and Protein A chromatography supports. In addition to natural Protein A purified from *Staphylococcus aureus*, there are also various recombinant constructs of Protein A typically produced in *E. coli*. Some of these recombinant Protein A's are essentially identical to natural Protein A. However, there are other unnatural

recombinant constructs of Protein A that have very significant structural differences when compared to natural Protein A. GE Healthcare sells one such unique construct of Protein A marketed as MabSelect SuRe™. Because of the very different structure of this protein the possibility exists that some antibodies for Protein A will recognize the various constructs of Protein A differently, resulting in quantitation inaccuracies. To address these differences, 2 separate kits have been developed so that labs using any form of Protein A can take advantage of the robust sample treatment and streamlined procedure of the Mix-N-Go Protein A ELISA format. The F600 kit is calibrated for natural and recombinant Protein A. This format is appropriate for most constructs of Protein A. The F610 kit uses natural recombinant Protein A calibrated against MabSelect Sure™. If you are using a recombinant form of Protein A with very significant structural differences from natural Protein A, you should use the Cat # F610 kit. If you are evaluating our Protein A assays for the very first time, we recommend use of the Cat # F600 or # F610 kits as these offer procedural advantages, improved robustness, and the ability to more accurately quantitate unnatural forms of Protein A when compared to Cygnus' older generation kits, (Cat # F050, F050H, F400 and F400Z). It is the responsibility of the end user to select the most appropriate kit and to qualify the kit with their sample types for acceptable accuracy and specificity.

The Mix-N-Go Protein A kits are designed to detect all currently marketed constructs of Protein A. They will eliminate most product antibody inhibition and provide accurate quantitation through the use of a carefully validated sample treatment step (See 'Limitations' section). These assays are designed to provide a simple to use, precise, and highly sensitive method to detect Protein A contamination to less than 100pg/mL in the presence of up to mg/mL quantities of humanized monoclonal antibodies. As such, these kits can be used as tools 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

The Mix-N-Go Protein A assays are a two-site immunoenzymetric assay. Samples containing Protein

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A are first diluted in the Mix–N-Go Sample Diluent provided with the kits. The Mix-N-Go Denaturing Buffer is then added and mixed to dissociate the Protein A from the product antibody. The samples are then reacted in microtiter strips coated with a polyclonal anti-Protein A capture antibody. A second anti-Protein A antibody labeled directly with Horse Radish Peroxidase (HRP) enzyme is simultaneously reacted forming a sandwich complex of solid phase antibody-Protein A:HRP labeled antibody. After a wash step to remove any unbound reactants, the strips are then reacted with tetramethylbenzidine (TMB) substrate. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of Protein A present in the sample. Accurate quantitation is achieved by comparing the signal of unknowns to Protein A standards assayed at the same time

Reagents & Materials Provided

Component	Product #
Anti-Protein A:HRP Chicken antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F601
Polyclonal Anti-Protein A coated microtiter strips 12x8 well strips in a bag with desiccant	F052*
Protein A Standards Recombinant Protein A in a protein matrix with preservative. 8 Standards at 0, 0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10 ng/mL. 1mL/vial	F603 or F613
Mix-N-Go Denaturing Buffer Citrate buffer with detergent and preservative. 1x12mL	F604
Mix-N-Go Sample Diluent Tris buffered saline with a protein matrix and preservative. 1x25mL	I600
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
Sample Treatment Plate Skirted 96 well PCR plate with adhesive foil seal	F402

*All components can be purchased separately except # F052.

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
Multichannel pipettor – 25µL, 50µL, and 100µL
Microtiter plate rotator (400 - 600 rpm)
Distilled water
1 liter wash bottle for diluted wash solution

Storage & Stability

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

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 antibody coated plate 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.

2. High Dose Hook Effect may be observed in samples with very high concentrations of Protein A. Samples greater than 20µg/mL may give absorbances less than the 10ng/mL standard. Hook effect is indicated when absorbance of the undiluted sample is less than the diluted samples. If a hook effect is possible, samples should be assayed over at least two dilutions.

Limitations

- Before reporting the Protein A contamination results, each laboratory should qualify that the kit

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and assay procedure utilized yield acceptable specificity, accuracy, and precision. A suggested protocol for this validation can be obtained by contacting our Technical Services Department or at our web site. In general, the most critical validation experiments involve spike & recovery and dilutional linearity/parallelism.

- Most mouse monoclonals, humanized monoclonals, and many human antibodies expressed in Chinese Hamster Ovary (CHO) cells or mouse hybridoma cell lines do not significantly cause inhibition and can be assayed at product concentrations of up to 5 mg/mL.

- Samples in concentrated strong acids can interfere in the assay by lowering the assay pH to below the optimal range of 7.0 to 7.5. The HRP labeled antibody is in a strong buffer designed to neutralize most samples back to the ideal assay pH range. If there is some doubt about the pH interference of your sample, you may conduct a simple test prior to performing the assay by adding 1 part of the denatured sample to 4 parts of the HRP conjugate and testing pH using paper pH indicator strips.

- Certain sample matrices and product antibodies may interfere in this assay. Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration, extremes of pH (<6.0 and >8.5), very high buffer molarity, or very high protein concentrations may give erroneous results. **For these reasons we recommend that you first establish acceptable recovery in your sample matrices by performing a spike recovery experiment.** This test can be very simply performed by diluting 1 part of the 10ng/mL standard supplied with the kit into 3 parts of your sample matrix which does not contain any or very low levels of Protein A. This diluted standard when assayed as an unknown should give a recovery value after correcting for any endogenous Protein A of ~2 to 3 ng/mL. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

- Very high IgG concentrations can inhibit the assay. This method has demonstrated excellent recovery in IgG concentrations up to 5 mg/mL. We recommend initially diluting each sample to a protein concentration of 1 mg/mL and Protein A in the analytical range of the curve as a starting point.

Assay Protocol

- 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. (A 630nm filter can be substituted for the 650nm if your instrument is so equipped.)

- All standards, controls and samples should be treated in exactly the same way. Assay all samples at least in duplicate.

- Maintain a repetitive timing sequence from well to well for all assay steps to ensure that all incubation times are the same for each well. Accomplish all steps as rapidly as possible to avoid "end of run" sequential process time differences that could cause systematic inaccuracies.

- Make a work list for each assay to identify the location of each standard, control, and sample.

- 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 is available in the 'Technical Help' section of our web site.

- The protocol specifies use of an approved microtiter plate shaker or rotator for the immunological steps. These can be purchased from most laboratory supply companies. If you do not have such a device, it is possible to incubate the plate without shaking however it will be necessary to extend the immunological incubation step in the plate by about one hour in order to achieve comparable results to the shaking protocol. **Do not shake during the 30-minute substrate incubation step, as this may result in higher backgrounds and worse precision.**

- For best results add Mix-N-Go Denaturing Buffer in the same direction as the replicates on the plate. For example, if the replicates are in A1 and A2 position the multichannel pipette horizontally when adding the Mix-N-Go Denaturing Buffer.

Procedural Modifications

- The assay is very robust such that assay variables like incubation times, and sample size 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 Technical Service for input on the best way to achieve your desired goals.

- Samples containing Protein A greater than 10ng/mL should **only** be diluted in the provided Mix-N-Go Sample Diluent (Cat # I600). Be sure to multiply diluted sample concentrations by the dilution factor when calculating the results.

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ATTACHMENT 5 (Continued)

Sample Treatment

Sample Treatment Procedure

1. Prepare initial sample dilutions as required prior to sample treatment. Transfer to the appropriate wells of the Sample Treatment Plate (STP), Cat # F402.

2. All subsequent dilutions to be assayed can be made in the STP using Sample Diluent, Cat # I600. Ensure final volume in every well is 100µL.

3. Add 100µL of the kit standards and controls to the wells.

4. Add 50µL of Mix-N-Go Denaturing Buffer, Cat #F604, to each well. Mix by pipetting up and down ~15 times. Use fresh tips for each addition.

5. Incubate on the bench for 5 – 10 minutes.

If you continue to have poor recovery after carefully following the procedures above, it may be necessary to further dilute your sample prior to assay using Mix-N-Go Sample Diluent Cat # I600. Dilution to the range of 1.0 to 0.1 mg/mL is usually sufficient to obtain acceptable recovery. Contact our very experienced Technical Service Department, if you have any problems with recovery.

Assay Protocol

Assay Protocol

1. Pipette 100µL of the anti-Protein A:HRP detection antibody (#F601) into each well of the antibody coated microtiter plate (#F052).

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

3. Cover & incubate on rotator at ~500rpm for 1 hour 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 (See 'Limitations' Section). 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.

Example Data

F600 Standard Curve			
Well #	Contents	Abs. at 450nm	Mean Abs.
1A	Zero Std	0.029	0.028
2A	Zero Std	0.027	
1B	0.16 ng/mL	0.080	0.078
2B	0.16 ng/mL	0.077	
1C	0.31ng/mL	0.138	0.136
2C	0.31ng/mL	0.135	
1D	0.63 ng/mL	0.257	0.260
2D	0.63 ng/mL	0.264	
1E	1.25ng/mL	0.488	0.489
2E	1.25ng/mL	0.491	
1F	2.5ng/mL	0.880	0.899
2F	2.5ng/mL	0.917	
1G	5ng/mL	1.800	1.790
2G	5ng/mL	1.780	
1H	10ng/mL	3.017	3.050
2H	10ng/mL	3.083	

F610 Standard Curve			
Well #	Contents	Abs. at 450nm	Mean Abs.
1A	Zero Std	0.024	0.024
2A	Zero Std	0.024	
1B	0.16 ng/mL	0.060	0.060
2B	0.16 ng/mL	0.059	
1C	0.31ng/mL	0.095	0.094
2C	0.31ng/mL	0.094	
1D	0.63 ng/mL	0.145	0.155
2D	0.63 ng/mL	0.164	
1E	1.25ng/mL	0.311	0.311
2E	1.25ng/mL	0.312	
1F	2.5ng/mL	0.603	0.616
2F	2.5ng/mL	0.630	
1G	5ng/mL	1.182	1.177
2G	5ng/mL	1.172	
1H	10ng/mL	2.461	2.431
2H	10ng/mL	2.400	

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Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 0.5 ng/mL. CVs for samples < 0.5 ng/mL may be greater than 10%.
- It is recommended that each 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 your product antibody. These controls can be aliquoted into single use vials and stored frozen for long-term stability.**

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. A more detailed copy of this "Qualification Summary" report can be obtained by request or by accessing our web site at www.cygnustechnologies.com. This qualification is generic in nature and is intended to supplement but not replace certain user and product specific qualification and validation that should be performed by each laboratory. At a minimum, each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your samples types containing Protein A within or above the analytical range of this assay should be evaluated for dilutional linearity to insure that the assay is accurate and does not suffer from "Hook Effect". 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 on-line at our web site.

Precision

The data below show both intra (n=16 replicates) and inter-assay (n=2 assays) coefficients of variation (%CVs) for 3 control samples in the low, middle, and upper range of the standards. Each laboratory is encouraged to establish precision with its protocol using a similar study.

F600 Kit		
Intra-assay		
# of tests	Target (ng/mL)	%CV
16	7.5	5.8
16	3	6.4
16	0.5	3.6
Inter-assay		
# of assays	Target (ng/mL)	%CV
2	7.5	6.3
2	3	8.8
2	0.5	6.7

F610 Kit		
Intra-assay		
# of tests	Target (ng/mL)	%CV
16	7.5	5.6
16	3	5.6
16	0.5	7.1
Inter-assay		
# of assays	Target (ng/mL)	%CV
2	7.5	6.1
2	3	7.4
2	0.5	8.2

Sensitivity

The lower limit of detection (**LOD**) is defined as that concentration corresponding to a signal three standard deviations above the mean of the zero standard. The LOD is ~50 pg/mL. The lower limit of quantitation (**LLOQ**) is defined as the lowest concentration at which recovery is within 20% of the nominal level and the repeatability is within 20% of the CV. The LLOQ of these methods is 100pg/mL.

Spike & Recovery/Interference Studies

Each user should qualify that their sample matrices and product itself yield accurate recovery. This experiment can be performed by spiking the 10ng/mL standard provided with this kit, into the sample in question. For example, we suggest adding 1 part of the 10 ng/mL standard to 3 parts of the test sample. This yields an added spike of 2.5 ng/mL. Any endogenous Protein A from the sample itself, determined prior to spiking and corrected for the 25% dilution of that sample, should 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%.

Various buffer matrices have been evaluated by spiking known amounts of Protein A. Because these assays are 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) or salt concentration as well as certain detergents can cause under-recovery. In some cases, high concentrations of the product antibody may also cause a negative interference. While the kits have been designed to overcome such interferences, your product antibodies should be evaluated for any negative inhibition before reporting results as described in the "Limitations" section. If you encounter product antibody interference, contact Cygnus Technologies Technical Services Department for advice on how to solve this problem. When detection sensitivity limits allow, simple dilution of the inhibitory product antibody in our recommended Mix-N-Go Sample Diluent Cat # I600 will usually overcome the interference.

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ATTACHMENT 5 (Continued)

Specificity/Cross-Reactivity

The F600 kit will detect natural *Staphylococcal* and various commercially available recombinant Protein A constructs. The F610 kit will detect unnatural Protein A constructs such as MabSelect SuRe™. However, you may want to test your source of Protein A for recovery to ensure accurate quantitation by these kits. If recovery is unacceptable, contact our Technical Services Department for guidance. Substitution of standards made with your source of Protein A in place of the kit standards may be a solution.

Hook Capacity

Very high concentrations of Protein A were evaluated for the hook effect. At concentrations exceeding 20,000 ng/mL, the apparent concentration of Protein A may read less than the 10ng/mL standard. Samples yielding signals above the 10ng/mL standard or suspected of having concentrations in excess of 20,000 ng/mL should be assayed diluted.

Ordering Information/ Customer Service

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

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

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ATTACHMENT 6
Cygnus Technologies Manual Plate Washing

CYGNUS TECHNOLOGIES

Washing Technique for Microtiter Wells

Our experience has shown that the most frequent cause of customer problems in the performance of ELISA is due to plate washing equipment and technique. While the goal of washing is to remove unbound reactants, many technicians fail to understand that the washing step can also dissociate antibody bound reactants and can do so in a highly variable manner. Our experience has shown that automated plate washers and related multi-channel hand-held vacuum aspiration devices give inferior assay performance relative to the manual procedure described below. Some plate washers may leave behind very small but still significant liquid causing variable and higher assay background than can be achieved manually. In an effort to reduce carryover, many washers can be too aggressive in the physics of washing and aspirating and will actually variably dissociate antibody bound analyte resulting in both lower absorbance and higher %CVs. For these reasons we strongly recommend that users use the manual procedure detailed below for optimal assay performance.

Equipment Required: All that is required for equipment is a wash/squirt bottle and low lint absorbent paper. We suggest that the narrow portion of the tip of the squirt bottle be cut off to give the largest possible orifice so that the flow will be generous and gentle. This procedure is best done over a large sink since it can be a little messy.

Step 1. Dumping the liquid from the plate:
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 you 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. When done properly you should not get any liquid on your fingers or on the outside of the strip wells or plate holder. Repeat the dumping motion a second time.

Step 2. Blotting and tapping the plate:
Immediately blot the upside down plate onto the blotting paper. Move the plate to an unused section of the blotting paper. Firmly tap the plate 3 times over unused areas of the paper. Do not bang the plate too hard in a misguided effort to remove all of the residual liquid, as this may cause variable dissociation due to transferring of unequal amounts of shock energy across the plate.

Step 3. Washing:
Use the squirt bottle to fill all wells staring at the front of the plate and working to the back. Fill to overflowing with the 20 fold diluted wash solution provided with the kit.
➔ **Do not** use any other wash buffer formulation as it may negatively impact the performance of the kit.
➔ **Do not** worry about overflowing the wells as you will be wiping off the bottom

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Cygnus Technologies Manual Plate Washing

of the wells before adding substrate.
➔**Do not** allow the wash solution to soak in the wells.

Immediately dump & tap the plate as described in Steps 1 & 2 as soon as the last well is filled.

Repeat the washing procedure 3 more times for a total of 4 washes. With the 2nd & 4th washes start by adding the wash solution from the back to the front of the plate. This insures that the total dwell time of wash solution in the wells will be essentially the same for all wells. Additional wash steps should not be necessary and in fact may dissociate specific bound analyte and actually reduce assay sensitivity.

After the last wash, blot and tap as described in Steps 1 & 2. Let the plate rest upside down for about 20 seconds to drain. Firmly tap the plate again 4 times rotating the plate 180º in your hand between each tap. This rotation ensures that the ends of the plate receive on average the same energy and impact from the banging.

Step 4. Wiping the plate:
Wipe the bottom outside of all wells with clean absorbent paper to remove any wash liquid from the overfilling and tapping. Wells are now ready to have the substrate added to them. Add substrate immediately after washing.
➔**Do not** let the wells dry out or enzymatic activity will be lost.
➔**Do not** add substrate near the sink location where your dumping and banging have taken place since the washing procedure can generate aerosols that could re-contaminate the wells or your substrate.
Watch the video Washing Technique for Microtiter Plate ELISA Video to get the exact technique.