



Table of Contents

1.0	Purpose.....	1
2.0	Scope	1
3.0	Authority and Responsibility	1
4.0	Supplies and Reagents	1
5.0	General Comments.....	2
6.0	Preparation	2
7.0	Procedure.....	4
8.0	Data Analysis and Acceptable Criteria.....	5
9.0	Documentation	6
10.0	References and Related Documents.....	7
11.0	Attachments.....	7
12.0	Change Summary	7

1.0 Purpose

This Standard Operating Procedure (SOP) describes the use of the Cygnus, Inc. ELISA kit for Total Mouse IgG quantitation.

2.0 Scope

This SOP applies to PA personnel who will perform the Total Mouse IgG ELISA.

Note: It is required that new (not previous 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 test.

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 Biopharmaceutical Quality Assurance (BQA) is responsible for quality oversight of this procedure.

4.0 Supplies and Reagents

- 4.1 *Total Mouse IgG* ELISA kit, BDP PN 30405, Cygnus Technologies, Catalog Number F049.
- 4.2 Sample Diluent Buffer, BDP PN 30406 Cygnus Technologies, Catalog Number I028.

- 4.3 Calibrated multi-channel pipettor.
- 4.4 Calibrated pipettors 2-200 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, 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 Avoid contamination of the workspace when handling Total Mouse IgG 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) or very high protein concentrations may give erroneous results. A buffer blank is required to be submitted with the sample.
- 5.6 The antibodies used in this kit are mouse immunoglobulin heavy and light chain specific. They do not cross react significantly with immunoglobulin from other species, but they will cross react to varying degrees with other mouse immunoglobulines such as all IgG isotypes, IgA, and IgM. The presence of high concentrations of mouse IgM and IgA immunoglobulin types in the sample can interfere in their ability of this kit to quantitate mouse IgG. Cygnus provides kits for those immunoglobulines.

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 = 0-20 ng/mL.

6.1.1 Prepare the test article immediately before use, undiluted and at 1:5 and 1:50 dilution using the Sample diluent (see 4.2). If the absorbance results indicate that the value for the 1:50 dilution is greater than 20 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 μ L aliquots), add 100 μ L test article to 400 μ L 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 μ L of the 1:5 diluted test article to 450 μ L diluent.

6.1.2 Prepare the spiked test article and a spiked buffer control. Spiked samples can be prepared as follows:

- A spiked sample can be made up directly in the microtiter strip well. For a spiked concentration of 5 ng/mL, do a 4x dilution into the well using the 20 ng/mL Cygnus, Inc. Total Mouse IgG standard. For example, add 12.5 μ L of the 20 ng/mL standard directly into the well containing 37.5 μ L 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 μ L of the 20 ng/mL standard directly into the well containing 37.5 μ L of the buffer provided by the requestor. This produces a 5 ng/mL concentration of Total Mouse IgG 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, 0.25, 1, 4, and 20 ng/mL are included in the Cygnus Total Mouse IgG kit. In order to increase the number of points on the standard curve, at least one more standard, a 2 ng/mL or a 10 ng/mL standard, is prepared fresh immediately before use. For example:

- To make a 2 ng/mL standard, do a 1:1 dilution with the 4 ng/mL standard. Add 25 μ L of the 4 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 10 ng/mL standard, do a 1:1 dilution with the 20 ng/mL standard. Add 25 μ L of the 20 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 2 ng/mL or 10 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 DirectQ 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 200 μ L of **anti-mouse immunoglobulin: alkaline phosphatase** into each well.
- 7.3.1 Pour contents of **anti-mouse immunoglobulin: alkaline phosphatase** 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 2 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 stripholder 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 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 200 µL of the **PNPP substrate** as in steps 7.3.1 - 7.3.2.

Note: If the substrate has a distinct yellow color prior to the assay, it may have been contaminated. If this appears to be the case, read 200 µL of substrate against a water blank. If the absorbance is greater than 0.4, obtain a new substrate (substrates are not kit specific) so the sensitivity of the assay will not be compromised.

- 7.8 Place the microtiter plate into a new Ziploc bag. Incubate at room temperature for 60 ± 2 minutes.
- 7.9 Remove the plate from the Ziploc bag.
- 7.10 Read absorbance at 405/492 nm, blanking on the zero standard. Refer to **SOP 22100, Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser** or analysis of the data.

8.0 Data Analysis and Acceptable Criteria

After the Ascent Total Mouse IgG 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 'fr-s-bdp-vlan' (s:)."
- 8.3 Click on the "PA" folder. Click on the "ELISA Results" folder. Select the specific ELISA test being analyzed; for example, BSA Template, Murine IgG Template, *E.coli* Host Cell Protein Template etc.
- 8.4 Fill in the top portion of the summary sheet with all appropriate information, including QC number, Analyst, Date, Sample ID, Lot Number, Kit Lot Number, and Expiration Date.



- 8.5 Fill in the "Total Mouse IgG 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 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 considerations of dilutional data where the assay value before dilution correction falls below two times the LOQ of the assay (1 ng/mL for the Total Mouse IgG 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 Total Mouse IgG 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 < 1ng/mL.

- 9.3 Record all solution preparation on Form 22123-01, Total Mouse IgG ELISA Preparation and include with the QC Test Request Form.

10.0 References and Related Documents

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

SOP 22702 *Solutions Used in Process Analytics*

Form 22123-01 Total Mouse IgG ELISA Preparation

Cygnus Technologies Total Mouse IgG Kit Instructions (Attachment 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 Total Mouse IgG ELISA Results

11.3 Attachment 3 Sample EXCEL Summary Worksheet

11.4 Attachment 4 Cygnus Technologies Inc. Total Mouse IgG ELISA Kit Insert



Attachment 1
Sample ELISA Work List

H	G	F	E	D	C	B	A	↑
20 ng/ml Standard	10 ng/ml Standard	4 ng/ml Standard	2 ng/ml Standard	1 ng/ml Standard	0.25 ng/ml Standard	0 ng/ml Standard	BLANK 0ng/ml Std	1
20 ng/ml Standard	10 ng/ml Standard	4 ng/ml Standard	2 ng/ml Standard	1 ng/ml Standard	0.25 ng/ml Standard	0 ng/ml Standard	BLANK 0ng/ml Std	2
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED 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 Total Mouse IgG ELISA Results

Ascent Software

Curve Fit1

6/15/04 4:46 PM

Session: C:\ASCENT\050604.SEE
Instrument: EMS Reader MF V2.9-0D
User name: XXXXXXXXXX
Started at: 5/6/04 4:45:22PM
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			pool	pool	abx	abx	Q	Q
B	Cal1	Cal1			spool	spool	sabx	sabx	sQ	sQ
C	Cal2	Cal2			pool	pool	A	A	Q	Q
D	Cal3	Cal3			spool	spool	sA	sA	sQ	sQ
E	Cal4	Cal4	nc	nc	abx	abx	A	A	Q	Q
F	Cal5	Cal5	snc	snc	sabx	sabx	sA	sA	sQ	sQ
G	Cal6	Cal6	pool	pool	abx	abx	A	A		
H	Cal7	Cal7	spool	spool	sabx	sabx	sA	sA		

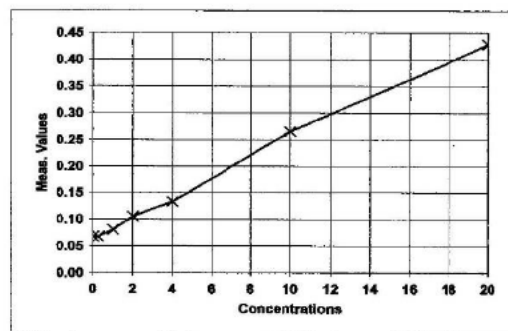
Source data for calibrators Sheet: Measure1, Assay: Assay1 and for samples Sheet: Measure1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	0.065	0.060			0.257	0.273	0.064	0.067	0.246	0.344
B	0.065	0.069			0.312	0.285	0.119	0.126	0.290	0.260
C	0.071	0.062			0.101	0.109	0.753	0.775	0.156	0.145
D	0.080	0.080			0.171	0.162	0.658	0.673	0.236	0.216
E	0.102	0.107	0.066	0.066	0.255	0.205	0.162	0.150	0.075	0.090
F	0.133	0.131	0.125	0.127	0.271	0.266	0.229	0.206	0.137	0.145
G	0.261	0.269	0.861	0.937	0.111	0.116	0.072	0.069		
H	0.413	0.441	0.788	0.727	0.179	0.177	0.139	0.135		

Sheet: Measure1, Assay: Assay1

Name	Meas.	Conc.
Cal1	0.065	0.00
	0.069	
	0.067	
Cal2	0.071	0.25
	0.062	
	0.067	
Cal3	0.080	1.00
	0.080	
	0.080	
Cal4	0.102	2.00
	0.107	
	0.105	
Cal5	0.133	
	0.131	

	Conc.	Meas.	CalcConc.	Residual
Cal1	0	0.067	0.27778	-0.27778
Cal2	0.25	0.067	0.25	1.3878e-16
Cal3	1	0.080	1	2.2204e-16
Cal4	2	0.105	2	0
Cal5	4	0.132	4	0
Cal6	10	0.265	10	0
Cal7	20	0.427	20	0





Attachment 3
Sample EXCEL Summary Worksheet

Summary of Total Mouse IgG ELISA Results

QC Number: QC-00000 **Analyst:** K.Leib **Date:** 4/19/2010

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

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

Total Mouse IgG Concentration (ng/mL)						
Acceptable Criteria: %CV <25% for samples >4ng/mL						
<u>Dilution Factor</u>	<u>Measured Concentration</u>		<u>Corrected Concentration</u>		<u>Average</u>	<u>%CV</u>
	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 "Mouse IgG Spiking Solution" (ng/ml): 20.0

Correlation Coefficient of Standard Curve: point to point

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

	Expected	Found	%Recovery	%CV
Avg. Positive Control (ng/ml):	10.00	10.05	100.50%	7.88
Spiked Buffer Control (ng/mL)	5.00	5.20	104.00%	8.15

Dilution Linearity

<u>Dilution Factor</u>	<u>Average Dilution Corrected Concentration</u>	<u>Percent Change in Dilution Corrected Concentration from Previous Value</u>
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% \pm 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 = $\frac{((\text{Spiked Conc} \times 0.050 \text{ mL}) - (\text{Unspiked Conc} \times 0.0375 \text{ mL}))}{(\text{Spike Conc} \times 0.0125 \text{ mL})} \times 100\%$

Analyst/Date: _____

Reviewed By/Date: _____

Attachment 4

Cygnus Technologies Inc. Total Mouse IgG ELISA Kit Insert



Total Mouse IgG Immunoglobulin Assay

Immunoenzymetric Assay for the Measurement of Total Mouse IgG Immunoglobulin Catalog # F049

Intended Use

This kit is intended for use in quantitating total mouse IgG. The antibodies used in this kit are specific for mouse immunoglobulins and will not cross react significantly with most immunoglobulins from other species. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

Monoclonal antibodies are used in many applications such as diagnostic or therapeutic agents as well as affinity supports for immunoaffinity purification of various products. These applications frequently require a sensitive assay to accurately quantitate the concentration of these antibodies in complex sample matrices. The antibodies used in this kit are mouse immunoglobulin heavy and light chain specific. They do not cross react significantly with immunoglobulin from other species but they will cross react to varying degrees with other mouse immunoglobulins such as all IgG isotypes, IgA, and IgM. The presence of high concentrations of mouse IgM and IgA immunoglobulin types in the sample can interfere in the ability of this kit to quantitate mouse IgG. *Cygnus Technologies* provides kits for IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM immunoglobulins. See ordering information on the last page.

Principle of the Procedure

The Mouse IgG assay is a two-site immunoenzymetric assay. Samples containing mouse IgG are reacted in microtiter strips coated with an affinity purified capture antibody. A second alkaline phosphatase enzyme labeled goat anti-mouse immunoglobulin antibody is reacted, forming a sandwich complex of solid phase antibody-mouse immunoglobulin-enzyme labeled antibody. The microtiter strips are then washed to remove any unbound reactants. The substrate p-nitrophenyl phosphate (PNPP) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of mouse IgG present. Accurate quantitation is achieved by comparing the signal of unknowns to IgG standards assayed at the same time.

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 absorbance at 405nm is greater than 0.4.

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

Reagents & Materials Provided

Component	Product #
Anti-Mouse IgG:Alkaline Phosphatase Affinity purified goat antibody conjugated to alkaline phosphatase in a protein matrix with preservative. 1x22mL	F076
Anti-Mouse IgG coated microtiter strips 12x8 well strips in a bag with desiccant	F075*
Mouse IgG Standards Mouse IgG in a bovine protein matrix with preservative. Standards at 0, 0.25, 1, 4, and 20 ng/mL. 1mL/vial	F081
PNPP Substrate p-nitrophenyl phosphate in a Diethanolamine buffer with preservative. 1x22mL	F008
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	F004

*All components can be purchased separately except # F075.

Materials & Equipment Required But Not Provided

Microtiter plate reader spectrophotometer with dual wavelength capability at 405 & 492nm. (If your plate reader does not provide dual wavelength analysis you may read at just the 405nm wavelength.)

Pipettors - 50µL and 100µL

Repeating or multichannel pipettor - 100µL

Microtiter plate rotator (150 - 200 rpm)

Sample Diluent (recommended Cat # 1028)

Distilled water

1 liter wash bottle for diluted wash solution

Precautions

* For Research or Manufacturing use only.

* At the concentrations used in this kit, none of the reagents are believed to be harmful.

* This kit should only be used by qualified technicians.



Attachment 4 (Continued)

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 manual operated vacuum aspiration devices for washing plates as these may result in lower specific absorbances, higher non-specific absorbance, and more variable precision. The manual wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision. If duplicate CVs are poor or if the absorbance of the 0 standard minus a substrate blank is greater than 0.15, evaluate plate washing procedure for proper performance.

2. High Dose Hook Effect may be observed in samples with very high concentrations of mouse immunoglobulin. Samples greater than 20µg/mL may give absorbances less than the 20ng/mL standard. Hook effect is indicated when absorbance of the undiluted sample is less than the diluted samples. If hook effect is possible, samples should also be assayed diluted.

3. When dilution of samples is required dilution should be performed in a diluent validated to yield acceptable background and not contaminated with mIgG. The diluent should also give acceptable recovery when spiked with known quantities of mIgG. The preferred diluent is our Cat# I-028 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 I-028 its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for recovery by using them to dilute the 20ng/mL standard, as described in the "Limitations" section below.

4. If the substrate has a distinct yellow color prior to performing the assay it may have been contaminated. If this appears to be the case read 200µL of substrate against a water blank. If the absorbance is greater than 0.4 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised. The PNPP substrate is very sensitive to environmental contamination. Do not leave bottle open or at room temperature for longer than is needed. Only remove as much reagent as is needed for your assay run and do not return any unused substrate back into the substrate bottle. Additional substrate can be purchased separately as Cat # F008.

Limitations

* The antibodies used in this kit cross-react with mouse IgM & IgA at approximately 10% on a molar basis.

* Certain sample matrices 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 dilution/recovery experiment.** This test can be very simply performed by diluting 1 part of the 20ng/mL standard supplied with the kit into 4 parts of your sample matrix that does not contain any or very low levels of mIgG. This diluted standard when assayed as an unknown should give a recovery value after correcting for any endogenous mIgG of 3 to 5 ng/mL. 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. Increasing incubation time for the PNPP substrate step will in general increase absorbances proportionately for all wells. For example, doubling the substrate step time from 60 minutes to 120 minutes will double all ODs. 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 the use of an approved microtiter plate shaker or rotator 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 first immunological incubation step by about 60 minutes to achieve comparable results to the 2 hour shaking protocol. **Do not shake during the 60 minute substrate incubation step as this may result in higher backgrounds and worse precision.**

* Bring all reagents to room temperature.

* Set-up plate spectrophotometer to read dual wavelength at 405nm for the test wavelength and 492nm 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.

* 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 yellow color prior to the assay it may have been contaminated. If this appears to be the case read 200µL of substrate against a water blank. If the absorbance is greater than 0.4 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.



Attachment 4 (Continued)

* Samples containing mIgG greater than 20ng/mL should be diluted in an appropriate diluent. (See Procedural Note # 2) Be sure to multiply diluted sample concentrations by the dilution factor when calculating the results.

* 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.

Assay Protocol

1. Pipette 50 μ L of standards, controls and samples into wells indicated on work list.
2. Pipette 200 μ L of Anti-Mouse IgG:Alkaline Phosphatase (#F076) into each well.
3. Cover & incubate on rotator at ~ 180rpm for 2 hours at room temperature, 24°C \pm 4°.
4. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding well from a squirt bottle or by pipetting in ~350 μ 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 200 μ L of PNPP substrate (#F008).
6. Incubate at room temperature for 60 minutes. DO NOT SHAKE.
7. Read absorbance at 405/492nm blanking on the Zero standard.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL. (See Limitations section above). 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.

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 < 1ng/mL may be greater than 10%.

* For optimal performance the absorbance of the substrate when blanked against water should be < 0.4.

* 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 405nm	Mean Abs.	ng/mL Mouse IgG
1A	Zero Std	0.000		
1B	Zero Std	0.002	0.001	
1C	0.25ng/mL	0.027		
1D	0.25ng/mL	0.026	0.027	
1E	1ng/mL	0.103		
1F	1ng/mL	0.100	0.102	
1G	4ng/mL	0.485		
1H	4ng/mL	0.495	0.490	
2A	20ng/mL	1.757		
2B	20ng/mL	1.739	1.748	
2C	sample 1	0.005		
2D	sample 1	0.010	0.008	<0.2ng
2E	sample 2	0.100		
2F	sample 2	0.105	0.103	1ng

Ordering Information/ Customer Service

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

www.cygnustechnologies.com

For other mouse immunoglobulin kits please specify the following catalog numbers:

Assay	Catalog #
IgG1	F045
IgG2a	F046
IgG2b	F047
IgM	F090