CYGNUS TECHNOLOGIES
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Mouse IgG2b Immunoglobulin Assay

Immunoenzymetric Assay for the Measurement of Mouse IgG2b Catalog # F047

Intended Use

This kit is intended for use in quantitating mouse IgG2b. The antibodies used in this kit are specific for mouse IgG2b and will not cross react significantly with most immunoglobulins from other species or with other mouse immunoglobulins. 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 IgG2b specific. They do not cross react significantly with immunoglobulins from other species or with the other mouse immunoglobulins; IgG1, IgG2a, IgG3, IgA or IgM. *Cygnus Technologies* provides kits for IgG1, IgG2a, IgG3, IgM and total mouse immunoglobulin. See ordering information on the last page.

Principle of the Procedure

The Mouse IgG2b assay is a two-site immunoenzymetric assay. Samples containing mouse IgG2b are reacted in microtiter strips coated with an affinity purified anti-IgG2b capture antibody. A second horseradish peroxidase (HRP) enzyme labeled goat anti-mouse IgG2b antibody is reacted forming a sandwich complex of solid phase antibody-mouse immunoglobulin-enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactants. The substrate tetramethylbenzidine (TMB) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of mouse IgG2b present. Accurate quantitation is achieved by comparing the signal of unknowns to IgG2b standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-Mouse IgG2b:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	**F159**
Anti-Mouse IgG2b coated microtiter strips 12x8 well strips in a bag with desiccant	**F164***
Mouse IgG2b Standards Mouse IgG2b in a bovine protein matrix with preservative. Standards at 0, 1, 4, 12, 35, and 100ng/mL. 1 mL/vial	**F079**
Stop Solution 0.5M sulfuric acid. 1x12mL	**F006**
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12mL	**F005**
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	**F004**

*All components can be purchased separately except # F164.

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on 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.
- 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 (400 - 600 rpm)

- Sample Diluent (recommended Cat # I028)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H_2SO_4. Avoid contact with eyes, skin, and clothing.
- 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 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 is greater than 0.2, 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 50µg/mL may give absorbances less than the 100ng/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 qualified to yield acceptable background and not contaminated with mIgG2b. The diluent should also give acceptable recovery when spiked with known quantities of mIgG2b. 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 100ng/mL standard, as described in the following "Limitations" section.

Limitations

- 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), 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 100ng/mL standard supplied with the kit into 4 parts of your sample matrix that does not contain any or very low levels of mIgG2b. This diluted standard when assayed as an unknown should give a recovery value after correcting for any endogenous mIgG2b of 15 to 25 ng/mL. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

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.

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 the use of an approved orbital microtiter plate shaker or rotator for the immunological step. These can be purchased from most laboratory supply companies. If you do not have such a device it is possible to incubate the plate without shaking however, it will be necessary to extend the first immunological

incubation step by about 30 minutes to achieve comparable results to the 1-hour 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, (NaN_3) which will destroy the HRP activity of the conjugate and could result in the under-estimation of Mouse IgG2b 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. If your plate reader does not have a 650nm filter it is acceptable to read at 450nm only.
- All standards, controls and samples should be assayed 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.
- 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 100µL of substrate plus 100µL 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.
- Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site. In addition, a video demonstration of proper plate washing technique is available in the 'Technical Help' section of our web site.

Assay Protocol

1. Pipette 50µL of standards, controls and samples into wells indicated on work list.

2. Pipette 100µL of anti-Mouse IgG2b:HRP (#F159) into each well.

3. Cover & incubate on orbital plate shaker at 400 – 600 rpm 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.

Example Data

Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.000	0.001
B1	Zero Std	0.002	
C1	1ng/mL	0.023	0.024
D1	1ng/mL	0.025	
E1	4ng/mL	0.103	0.106
F1	4ng/mL	0.108	
G1	12ng/mL	0.300	0.303
H1	12ng/mL	0.306	
A2	35ng/mL	0.762	0.765
B2	35ng/mL	0.767	
C2	100ng/mL	1.589	1.640
D2	100ng/mL	1.691	

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 1 – 100ng/mL. CVs for samples less than 1ng/mL may be greater than 10%.

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

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. This qualification is generic in nature and is intended to supplement but not replace certain user and product or sample specific qualification and qualification that should be performed by each laboratory. At a minimum each laboratory is urged to perform spike and recovery and dilutional linearity studies in their sample types. 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 qualification protocols can be obtained by contacting our Technical Services Department or 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 less than 0.3 ng/mL.

The lower limit of quantitation (**LOQ**) is defined as the lowest concentration where concentration coefficients of variation (CVs) are less than 20%. LOQ is ~0.5 ng/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=5 assays) precision were determined on 3 pools with low (1.50ng/mL), medium (25ng/mL), and high concentrations (75ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	4.8%	9.0%
Medium	3.8%	6.9%
High	5.1%	5.2%

Specificity/Cross-Reactivity

Cross reactivity in two site ELISAs can manifest itself as either a false increase in mouse IgG2b levels (positive cross reactivity) or as a false decrease in true mouse IgG2b (negative cross reactivity). Animal total immunoglobulin fractions at ~2mg/mL and/or undiluted sera from the various animal species shown below were tested for positive cross reactivity by assaying the sample as an unknown. Negative cross reactivity was evaluated by spiking 25 ng/mL of mouse IgG2b into each of the potential cross reactants and dividing the recovered value by 25 ng/mL. None of the materials below showed either type of cross reactivity except for mouse IgG which gave a percent cross reactivity of 0.001%. The antibodies used in this kit have been affinity purified to minimize cross reactivity but it is recommended that each user test their particular sample matrix material for cross reactivity in a similar experiment.

Animal Species	% Cross-Reactivity
Cat	Not detectable
Chicken	Not detectable
Cow	Not detectable
Dog	Not detectable
Goat	Not detectable
Guinea pig	Not detectable
Hamster, Syrian	Not detectable
Horse	Not detectable
Pig	Not detectable
Rabbit	Not detectable
Rat	Not detectable
Sheep	Not detectable

Other mouse immunoglobulins IgG1, IgG2a, IgG3, IgA and IgM were tested at 0.1mg/mL and showed no cross reactivity.

Recovery/ Interference Studies

Various buffer matrices have been evaluated by adding known amounts of the mouse IgG2b preparation used to make the standards in this kit. Because this assay is designed to minimize matrix interference most of these buffers yielded acceptable recovery defined as between 80-120%. In general, extremes in pH (less than 5.0 and greater than 8.5) as well as some detergents like SDS and Tween can cause under-recovery. Very high concentrations of certain proteins can also interfere in accurate detection of mouse IgG2b. Each user should qualify that their sample matrices yield accurate recovery by performing a similar experiment. For example, this experiment can be performed by diluting one part of the 100ng/mL standard provided with this kit into 4 parts of the sample matrix in question. Recovery should be on the order of 15 to 25 ng/mL mouse IgG2b. Consult *Cygnus Technologies* Technical Services if you have recovery problems in your matrix.

Hook Capacity

Increasing concentrations of mouse IgG2b greater than 50 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less that the 100 ng/mL standard was ~50 μg/mL.

Ordering Information/ Customer Service

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

Assay	Catalog #
IgG1	F045
IgG2a	F047
IgM	F090
IgG Total	F049

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

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