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TiterZyme ®EIA

mouse IgG_{2a} Isotyping

Enzyme Immunometric Assay Kit

Catalog No. 900-113

96 Well Kit

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Description

Assay Designs' mouse IgG_{2a} Isotyping TiterZyme® Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determintation of mouse IgG_{2a} in Tissue Culture Media and serum. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to mouse IgG immobilized on a microtiter plate to bind the mouse IgG in the standards or sample. A mouse IgG_{2a} Standard is provided in the kit. After a simultaneous incubation with a polyclonal antibody to anti-mouse IgG_{2a} conjugated to Horseradish peroxidase, which binds to the mouse IgG_{2a} captured on the plate, the excess reagents are washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of mouse IgG_{2a} in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

IgG_2 is divided into two subclasses; IgG_{2a} and IgG_{2b}. It is a glycoprotein which consists of two identical heavy chains (50kDal each) and two identical light chains (25 kDal each), to give a combined mass of approximately 150 kDal. The chains are held in place by covalent disulfide bonds. Each light chain contains two immunoglobulin (Ig) domains, while the heavy chains contain four Ig domains each. In the middle of each heavy chain is a relative varying portion called the “hinge region” which is unique to each IgG. This region allows for molecular flexibility and sets IgG_{2a} apart from its IgG counterparts. Properties of IgG_{2a} include neutralization of toxins and diffusion in the extracellular space[3].

Precautions

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1. Stop Solution 2 is a 1 normal (1N) hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.

Materials Supplied

1. **Goat anti-mouse IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0050**
   A plate using break-apart strips coated with polyclonal antibody specific to mouse IgG.
2. **Assay Buffer 13 Concentrate, 50 mL, Catalog No. 80-1604**
   Tris buffered saline containing proteins and detergents.
3. **mouse IgG_{2a} Conjugate, 6 mL, Catalog No. 80-1053**
   A blue solution of goat anti-mouse IgG_{2a} conjugated to Horseradish peroxidase.
4. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
   Tris buffered saline containing detergents.
5. **mouse IgG_{2a} Standard, 2 vials, Catalog No. 80-1052**
   Two vials containing 500 ng each of lyophilized mouse IgG_{2a}.
6. **TMB Substrate, 12 mL, Catalog No. 80-0350**
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. Ready to use. **Protect from prolonged exposure to light.**
7. **Stop Solution 2, 11 mL, Catalog No. 80-0377**
   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**
8. **mouse IgG_{2a} Isotyping Assay Layout Sheet, 1 each, Catalog No. 30-0187**
9. **Plate Sealer, 2 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the standard**, are stable at 4 °C until the kit's expiration date. The Standard **must** be stored in the original bottle at -20 °C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 50 µL and 1,000 µL.
3. Disposable test tubes for dilution of samples and standards.
4. Repeater pipets for dispensing 50 µL.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. Plate shaker.
8. Adsorbent paper for blotting.
9. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
10. Graph paper for plotting the standard curve.

Sample Handling

Assay Designs' TiterZyme® EIA is compatible with mouse IgG_{2a} samples in Tissue Culture Media and mouse serum. Samples diluted sufficiently into the proper diluent can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions.

Culture fluids and serum are suitable for use in the assay. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples in the majority of Tissue Culture Media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the Tissue Culture Media instead of Assay Buffer 13. There will be a small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of mouse IgG_{2a} in the appropriate matrix.

Samples must be stored frozen to avoid loss of bioactive mouse IgG_{2a}. If samples are to be run within 24 hours, they may be stored at 4 °C. Otherwise, samples must be stored frozen at -70 °C to avoid loss of bioactive mouse IgG_{2a}. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen sera should be brought to room temperature slowly and gently mixed by hand. Do not thaw samples in a 37 °C incubator. Do not vortex or sharply agitate samples.

High Dose Hook

The assay shows no "high dose hook" effect to 625 ng/mL of mouse IgG_{2a}. A sample spiked to contain 1,250 ng/mL read as 302 ng/mL. However, elevated levels of mouse IgG_{2a} above 625 ng/mL in the sample to be assayed (after any suggested dilution) may read outside the linear range of the assay.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**
9. **It is important that the matrix for the standards and samples be as similar as possible. Mouse IgG_{2a} samples diluted with Assay Buffer 13 should be run with a standard curve diluted in the same buffer. Serum samples should be evaluated against a standard curve run in Assay Buffer 13 while Tissue Culture samples should be read against a standard curve diluted in the same complete but non-conditioned media. See Reagent Preperation, step #2.**

Reagent Preparation

1. Wash Buffer

Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. Assay Buffer 13

Prepare the Assay Buffer 13 by diluting 50 mL of the supplied concentrate with 450 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

3. mouse IgG_{2a} Standards

Allow the lyophilized mouse IgG_{2a} standard solution to warm to room temperature. Add 1.0 mL of(standard diluent Assay Buffer 13 or Tissue Culture Media) to the lyophilized mouse IgG_{2a} vial and vortex. Wait 5 minutes and vortex again prior to use. Label six 12x75 mm glass tubes #1 through #6. Pipet 250 µL of standard diluent into tubes #1 through #6. Add 250 µL of reconstituted standard to tube #1 and vortex. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #6.

The concentration of mouse IgG_{2a} in tubes #1 through #6 will be 250, 125, 62.5, 31.3, 15.6 and 7.81 ng/mL respectively. See mouse IgG_{2a} Assay Layout Sheet for dilution details.

Reconstituted and diluted standards should be used within 60 minutes of preparation.

Discard any unused reconstituted standard and subsequent dilutions.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

Plates will require shaking on an orbital rotor at 500 rpm.

All standards, controls and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 50 µL of standard diluent (Assay Buffer 13 or Tissue Culture Media) into the S0 (0 pg/mL standard) wells.
3. Pipet 50 µL of Standards #1 through #6 into the appropriate wells.
4. Pipet 50 µL of the Samples into the appropriate wells.
5. Add 50 µL of blue Conjugate to each well, except the Blank.
6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour.
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µL of Substrate Solution into each well.
9. Incubate for 30 minutes at room temperature on a plate shaker.
10. Pipet 100 µL Stop Solution 2 to each well. This stops the reaction and the plates should be read immediately.
11. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

Calculation of Results

Several options are available for the calculation of the concentration of mouse IgG_{2a} in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of mouse IgG_{2a} can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Using linear graph paper, plot the Average Net OD for each standard versus mouse IgG_{2a} concentration in each standard. Approximate a straight line through the points. The concentration of mouse IgG_{2a} in the unknowns can be determined by interpolation.

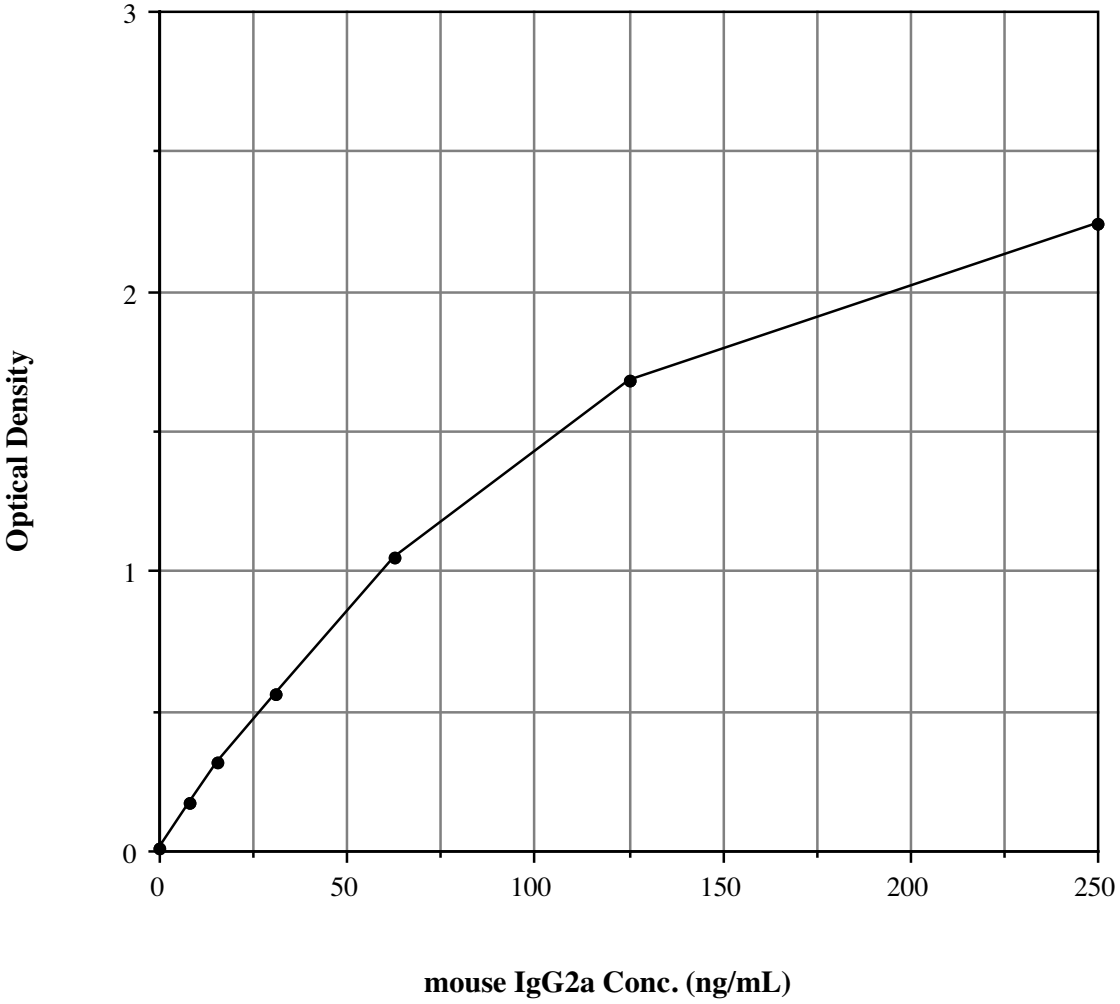
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Average OD	Net OD	**m IgG_{2a} (ng/mL)**
Blank	0.089		
S0	0.097	0.008	**0**
S1	2.327	2.238	**250**
S2	1.773	1.684	**125**
S3	1.133	1.044	**62.5**
S4	0.652	0.563	**31.3**
S5	0.405	0.316	**15.6**
S6	0.260	0.171	**7.81**
Unknown 1	1.543	1.454	**99.0**
Unknown 2	0.584	0.495	**26.2**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate mouse IgG_{2a} concentrations; each user must run a standard curve for each assay.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[4].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for fourteen (14) wells run with 0 ng/mL Standard, and comparing to the average optical density for fourteen (14) wells run with Standard #6. The detection limit was determined as the concentration of mouse IgG_{2a} measured at two (2) standard deviations from the 0 ng/mL Standard along the standard curve.

Mean OD for S0 =	0.011 $\pm$ 0.004 (36.7%)
Mean OD for Standard #6 =	0.207 $\pm$ 0.005 (2.7%)
Delta Optical Density (7.81 - 0 ng/mL) =	0.207 - 0.011 = 0.196
2 SD's of 0 ng/mL Standard =	0.008

Sensitivity = $\frac{0.008}{0.196} \times 7.81$ ng/mL = **318.8 pg/mL**

Linearity

A sample containing 219 ng/mL mouse IgG_{2a} was serially diluted 4 times 1:2 in the Assay Buffer 13 supplied in the kit and measured in the assay. The data was plotted graphically as actual mouse IgG_{2a} concentration versus measured mouse IgG_{2a} concentration.

The line obtained had a slope of 0.952 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of mouse IgG_{2a} and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of mouse IgG_{2a} in multiple assays run over 3 days (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of mouse IgG_{2a} determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	m IgG_{2a} (ng/mL)	Intra-assay % CV	Inter-assay % CV
Low	43.4	1.8	
Medium	69.8	1.2	
High	112	2.1	
Low	41.4		4.6
Medium	67.4		4.8
High	110		4.1

Cross Reactivities

The mouse IgG_{2a} Isotyping EIA kit is specific for mouse IgG_{2a}. There is 0.49% cross-reactivity with mouse IgG_3 and 0.12% cross-reactivity with mouse IgM. There is less than 0.01% cross-reactivity with rat IgG_{2a} and the following mouse protiens: IgG_1 and IgG_{2b}.

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard prepraration.

Mouse IgG_{2a} concentrations were measured in mouse serum and Tissue Culture Media. Mouse IgG_{2a} was spiked into the undiluted samples of these matrices which were then diluted with the appropriate diluent and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Mouse Serum	98.4	≥1:100,000
Tissue Culture Media	98.2	None

* See Sample Handling instructions on page 4 for details.

References

1. T. Chard,"An Introduction to Radioimmunoassay & Related Techniques 4th Ed.", (1990) Amsterdam: Elsevier.
2. P. Tijssen,"Practice & Theory of Enzyme Immunoassays", (1985) Amsterdam: Elsevier.
3. P. Parham,"The Immune System", (2000) New York: Garland Publishing.
4. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

LIMITED WARRANTY

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

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Material Safety Data Sheet (MSDS) available on our website or by fax.

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