

Mouse Anti-PEG IgM ELISA

Life Diagnostics, Inc., Catalog Number: PEGM-1

INTRODUCTION

Attachment of polyethylene glycol (PEG) chains to therapeutic biologic agents, a process referred to as PEGylation, prolongs the circulating half-life of the modified protein by slowing proteolytic degradation and by masking it from the immune system. However, it has been reported that repeated injections of PEGylated proteins can induce anti-PEG antibodies that increase the rate of clearance and decrease drug efficacy (accelerated blood clearance, or ABC phenomenon). To aid research in this important area, we have developed a mouse anti-PEG IgM ELISA kit.

PRINCIPLE OF THE ASSAY

The assay uses immobilized mono mPEGylated BSA (20 kDa PEG chain) as the capture antigen (coated on microtiter wells) and horseradish peroxidase (HRP) conjugated anti-mouse IgM antibodies for detection. Serum or plasma samples are diluted and incubated alongside standards in the microtiter wells for 1 hour. The wells are subsequently washed, and HRP conjugate is added and incubated for 30 minutes. Anti-PEG IgM molecules are thus sandwiched between immobilized PEG and the detection antibody conjugate. The wells are then washed to remove unbound HRP-labeled antibodies. TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow. Optical density is measured spectrophotometrically at 450 nm. The concentration of anti-PEG IgM is proportional to the absorbance at 450 nm and is derived from a standard curve.

This assay primarily detects antibodies directed against the polyoxyethylene backbone of PEG. Studies at Life Diagnostics, Inc, in mice and rabbits have demonstrated that most anti-PEG antibodies induced by immunization with PEGylated proteins are directed against the PEG backbone.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- PEG-BSA coated plate (12 x 8-wells) **Store at -20°C**
- Anti-Mouse IgM HRP Stock **Store at -20°C**
- Reference Stock¹ (lyophilized) **Store at -20°C**
- 20x HRP PEG Wash: PEGW50-20, 50 ml
- HRP PEG Diluent: PEGD50-1, 50 ml
- TMB: TMB11-1, 11 ml
- Stop Solution: SS11-1, 11 ml

Materials required but not provided:

- Pipettors and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm.
- Curve fitting software

STORAGE

The reference stock, HRP conjugate and the PEG-BSA coated plate should be stored at -20°C. All remaining kit components should be stored at 4°C. The microtiter plate should be kept in a sealed bag with desiccant. Kits will remain stable for six months from the date of purchase provided that the components are stored as described.

GENERAL INSTRUCTIONS/LIMITATIONS

1. Please read and instructions thoroughly before using the kit.
2. This kit is designed to measure anti-PEG IgM levels in serum collected ≈7 days after immunization with PEG. Serum collected at post-immunization times greater than 7 days may contain high levels of anti-PEG IgG that compete with anti-PEG IgM for the immobilized PEG, thereby causing interference.
3. All reagents should be allowed to reach room temperature (25°C) before use.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. Use only the wash solution and dilution buffer provided with the kit. PEG and PEGylated compounds are found in many buffers conventionally used in ELISA's and cannot be used with this kit.
6. Kits are validated using plate shakers set at 150 rpm and 25°C. Performance of the assay at lower temperatures and/or mixing speeds will likely result in lower absorbance values.
7. Optimal results are achieved if, at each step, reagents are pipetted into the wells of the microtiter plate within 5 minutes.

WASH SOLUTION PREPARATION

The wash solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water.

STANDARD PREPARATION

1. The mouse anti-PEG IgM standard is provided as a lyophilized stock. Reconstitute as described on the vial label.
2. Label 5 polypropylene or glass tubes as 100, 50, 25, 12.5, and 6.25 u/ml.
3. In the tube labeled 100 u/ml prepare the 100 u/ml standard as detailed on the stock vial label.
4. Dispense 250 µl of diluent into the remaining tubes.
5. Prepare a 50 u/ml standard by diluting and mixing 250 µl of the 100 u/ml standard with 250 µl of diluent in the tube labeled 50 u/ml.
6. Similarly prepare the 25, 12.5, and 6.25 u/ml standards by serial dilution.

SAMPLE PREPARATION

Studies at Life Diagnostics, Inc. indicate that anti-PEG IgM levels were undetectable in serum from control mice. However, in serum from PEG immunized mice, levels of 13,382 to 75,388 u/ml (47806 ± 18885 , mean \pm SD, $n = 10$) were found seven days after immunization with PEG-KLH. Levels will vary with the immunization protocol and the PEG carrier protein used. We suggest that samples initially be diluted 500-fold using the following procedure, but optimal

¹ Mouse anti-PEG IgM levels are measured in nominal units and are calibrated using pooled anti-PEG mouse serum prepared at Life Diagnostics, Inc.

dilutions must be determined empirically. A 500-fold dilution may be achieved as follows:

1. Dispense 48 μ l and 237.5 μ l of diluent into separate tubes.
2. Pipette and mix 2 μ l of the serum/plasma sample into the tube containing 48 μ l of diluent. This provides a 25-fold diluted sample.
3. Mix 12.5 μ l of the 25-fold diluted sample with the 237.5 μ l of diluent in the second tube. This provides a 500-fold dilution of the sample.
4. Repeat this procedure for each sample to be tested.

HRP CONJUGATE PREPARATION

Approximately 5 minutes before needed, dilute the HRP Conjugate stock with diluent (equilibrated to room temperature) as directed on the vial label.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 μ l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 150 rpm/25°C for 1 hour.
4. Aspirate the contents of the microtiter wells and wash the wells 5 times with 1x wash solution using a plate washer (400 μ l/well).
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash solution.
6. Add 100 μ l of diluted HRP conjugate into each well.
7. Incubate on an orbital micro-plate shaker at 150 rpm/25°C for 30 minutes.
8. Wash as detailed above.
9. Dispense 100 μ l of TMB reagent into each well.
10. Gently mix on an orbital micro-plate shaker at 150 rpm/25°C for 20 minutes.
11. Stop the reaction by adding 100 μ l of Stop Solution to each well.
12. Gently mix. It is important to make sure that all the blue color changes to yellow.
13. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

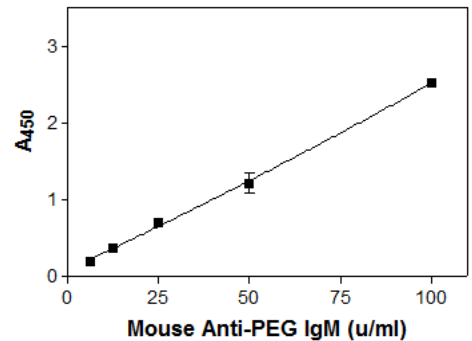
CALCULATION OF RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus concentration.
2. Fit the standard curve to a second order polynomial model and determine the concentration of the samples from the standard curve.
3. Multiply the derived concentration by the dilution factor to determine the actual concentration in the samples.
4. If the A_{450} values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y-axis against anti-PEG IgM concentrations on the X-axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns.

Anti-PEG IgM (u/ml)	A_{450}
100	2.519
50	1.214
25	0.699
12.5	0.375
6.25	0.196



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For technical assistance please email us at techsupport@lifediagnosics.com