Mouse Anti-PEG IgG ELISA PEGG-1-N

INTRODUCTION

The attachment of polyethylene glycol 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, repeated injections of PEGylated proteins can induce anti-PEG antibodies that increase the rate of clearance and thereby decrease efficacy (accelerated blood clearance, or ABC phenomenon). To aid research, we have developed a mouse anti-PEG IgG 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 IgG 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 45 minutes. Anti-PEG IgG molecules are thus sandwiched between immobilized PEG and the detection antibody conjugate. The wells are then washed to remove unbound HRP-labeled antibodies, and 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 IgG 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

Materials provided with the kit:

- PEG-BSA coated plate (12 x 8-wells) **Store at -20°C**
- Anti-Mouse IgG HRP Stock (lyophilized) **Store at -20°C**
- Reference Stock[1] (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 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 IgG levels in serum collected >14 days after immunization with PEG. Serum collected at post-immunization times less than 14 days may contain elevated levels of anti-PEG IgM that compete with anti-PEG IgG 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 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

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.

DILUENT

The diluent (PRGD50-1) is formulated for measurement PEG antibodies. It is supplied ready to use. DO NOT substitute other buffers.

STANDARDS

1. The mouse anti-PEG IgG standard is provided as a lyophilized stock. Reconstitute the stock as described on the vial label.
2. Label 6 polypropylene or glass tubes as 100, 50, 25, 12.5, 6.25 and 3.13 u/ml.
3. In the tube labeled 100 u/ml prepare the 100 u/ml standard as detailed on the stock vial label.

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

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, 6.25 and 3.13 u/ml standards by serial dilution.

HRP CONJUGATE

Approximately 15 minutes before needed, reconstitute the lyophilized HRP conjugate by adding 200 µl of diluent and mix gently. Dilute as described on the vial label to give the working conjugate solution. The reconstituted conjugate stock must be stored at -20°C in a sealed vial if future use is intended.

SAMPLES

Studies at Life Diagnostics, Inc. indicated that anti-PEG IgG levels were undetectable in serum from control mice. However, in serum from PEG-KLH immunized mice, levels of 12,900 to 133,100 u/ml (71056 ± 61394, mean ± SD, n = 8) were found twenty-five days after immunization. Levels will vary with the immunization protocol and the PEG carrier protein used. We suggest that samples initially be diluted 2000-fold, but optimal dilutions must be determined empirically. A 2000-fold dilution may be achieved as follows:

1. Dispense 98 µl and 341.25 µl of diluent into separate tubes.
2. Pipette and mix 2 µl of the serum/plasma sample into the tube containing 98 µl of diluent. This provides a 50-fold diluted sample.
3. Mix 8.75 µl of the 50-fold diluted sample with the 341.25 µl of diluent in the second tube. This provides a 2000-fold dilution.

PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards and diluted samples into the wells (we recommend testing in duplicate).
3. Incubate on a plate shaker at 150 rpm/25°C for 1 hour.
4. Aspirate the contents of the microtiter wells and wash the wells five times with 1x wash solution using a plate washer (400 µl/well).
5. Strike the wells sharply onto absorbent paper to remove all residual wash solution.
6. Add 100 µl of diluted HRP conjugate into each well.
7. Incubate on a plate shaker at 150 rpm/25°C for 45-minutes.
8. Wash as detailed above.
9. Dispense 100 µl of TMB into each well.
10. Incubate on a plate shaker at 150 rpm/25°C for 20-minutes.
11. Stop the reaction by adding 100 µl 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 five minutes[2].

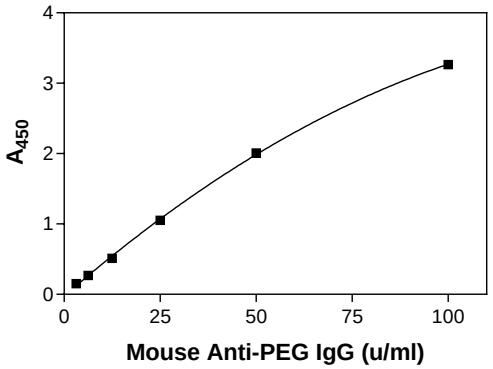
RESULTS

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 IgG 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 IgG (u/ml)	A_{450}
100	3.266
50	2.010
25	1.052
12.5	0.513
6.25	0.267
3.125	0.151



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[2] On some plate readers the A450 value of the high standard may be out of range. If that occurs, absorbance values for all wells may be read at 405 nm instead. Absorbance values will be lower, but this does not affect results.

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