

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). In our own studies, we find that a single injection of PEGylated protein induced high anti-PEG IgG titers (refer to the "Sample Preparation" section.) To aid research in this key area, we have developed a monkey 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-monkey IgG monoclonal antibody (LDI clone 6D11-2¹) for detection. Serum or plasma samples are diluted and incubated alongside standards in the microtiter wells for one hour. The wells are subsequently washed. 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-conjugate. 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, and 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, demonstrated that immunization of monkeys (n = 3) with mPEG-KLH induced anti-PEG IgG that was exclusively directed against the PEG backbone, not the terminal methoxy group.

MATERIALS

Materials provided with the kit:

- PEG-BSA coated plate (12 x 8-wells) **Store at -20°C**
- Anti-Monkey IgG HRP Stock (lyophilized) **Store at -20°C**
- Reference Stock² (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 understand the 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 (PEGD50-1) is formulated for measurement PEG antibodies. It is supplied ready to use. DO NOT substitute other buffers.

¹ Clone 6D11-2 recognizes rhesus, cynomolgus, baboon and human IgG

² Monkey anti-PEG IgG levels are measured in nominal units and are calibrated using pooled anti-PEG monkey serum prepared at Life Diagnostics, Inc.

STANDARDS

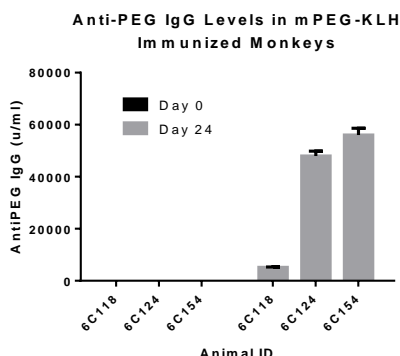
1. The monkey anti-PEG IgG standard is provided as a lyophilized stock. Reconstitute the stock as described on the vial label.
2. Label 7 polypropylene or glass tubes as 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 u/ml.
3. In the tube labeled 100 u/ml prepare the 100 u/ml standard as detailed on the stock vial label.
4. Dispense 250 μ l of diluent into the remaining tubes.
5. Prepare a 50 u/ml standard by diluting and mixing 250 μ l of the 100 u/ml standard with 250 μ l of diluent in the tube labeled 50 u/ml.
6. Similarly prepare the remaining standards by serial dilution.

SAMPLES

General Note: In studies at Life Diagnostics, Inc., we found that anti-PEG IgG levels were undetectable in serum from naïve monkeys. Twenty-four days after a single immunization with mPEG-KLH, levels increased to $36,406 \pm 27,336$ u/ml (mean \pm SD, n = 3, see figure below). Levels will vary with the immunization protocol and the PEG carrier protein used.

Optimal dilutions must be determined empirically. However, we suggest that samples initially be diluted 1000-fold using the following procedure.

1. Dispense 95 μ l and 294 μ l of diluent into separate polypropylene or glass tubes.
2. Pipette and mix 5 μ l of the serum sample into the tube containing 95 μ l of diluent. This provides a 20-fold diluted sample.
3. Dilute 6 μ l of the 20-fold diluted sample into the tube containing 294 μ l of diluent and mix. This provides a 1000-fold diluted sample.



HRP CONJUGATE

Approximately 15 minutes before needed, reconstitute the lyophilized HRP conjugate by adding 200 μ l 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.

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 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 μ l/well).
5. Strike the wells sharply onto absorbent paper to remove all residual wash solution.
6. Add 100 μ l 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 μ l 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 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 five minutes³.

RESULTS

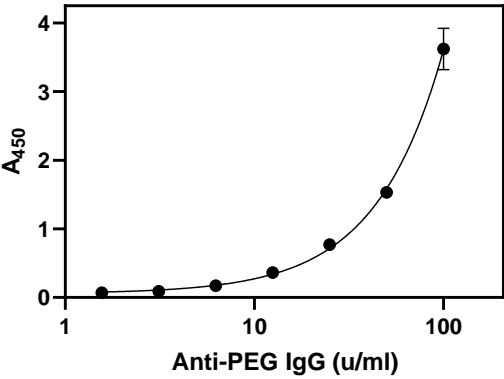
1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus \log_{10} of the concentration.
2. Fit the standard curve to a four-parameter logistic regression (4PL) equation (x axis = \log_{10} concentration) and determine the concentration of the samples from the standard curve (remember to derive the antilog).
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.

³ On some plate readers the A_{450} 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.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y-axis against log10 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	3.621
50	1.531
25	0.770
12.5	0.363
6.25	0.171
3.125	0.090
1.56	0.070



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