Rat Anti-Polyethylene Glycol (PEG) IgG ELISA

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). In order to aid research in this important area, we have developed a rat anti-PEG IgG ELISA kit.

PRINCIPLE OF THE ASSAY
The rat anti-PEG IgG test kit is based on a solid phase enzyme-linked immunosorbent assay (ELISA). 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-rat 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, 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., in mice and rabbits have demonstrated that the majority of 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 96-well Plate (provided as 12 strips of 8 wells). Store at -20°C.
- Anti-Rat IgG HRP Conjugate Stock, 50 µl. Store at -20°C.
- Reference Standard* (lyophilized), 1 vial. Store at -20°C.
- 20x HRP PEG Wash Solution, 50 ml
- HRP PEG Diluent, 50 ml
- TMB Reagent, 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:
- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-plate incubator/shaker mixing speed of ~150 rpm
- Plate washer
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional)

STORAGE
- The reference standard, HRP conjugate and the PEG-BSA coated plate should be stored at -20°C
- All remaining kit components should be stored at 2-8°C

The microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS
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 high 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 (18-25°C) before use.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc. suggest an initial sample dilution of 500 fold may be useful. The kit was validated using serum dilutions ≥50 fold.
5. 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 rat 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 and 6.25 µl.
3. In the tube labeled 100 µl prepare the 100 µl/ml standard as detailed on the stock vial label.
4. Dispense 350 µl of diluent into the remaining tubes.
5. Prepare a 50 µl/ml standard by diluting and mixing 350 µl of the 100 µl/ml standard with 350 µl of diluent in the tube labeled 50 µl/ml.
6. Similarly prepare the 25, 12.5 and 6.25 u/ml standards by serial dilution.

**SAMPLE PREPARATION**

General Note: Studies at Life Diagnostics, Inc. indicated that anti-PEG IgG levels were undetectable in serum from control rats. However, in serum from PEG-KLH immunized rats, levels of 1.544 to 19.994 u/ml (11726 ± 6636, 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 using the following procedure for each sample to be tested but optimal dilutions must be determined empirically. A 2000 fold dilution may be achieved as follows:

1. Dispense 48 μl and 332.5 μl of diluent into separate tubes.
2. Pipette and mix 2 μl of the serum/plasma sample into the tube containing 48 μl of diluent. This provides a 25 fold diluted sample.
3. Mix 17.5 μl of the 25 fold diluted sample with the 332.5 μl 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 μl of standards and diluted samples into the wells (we recommend that samples be tested in triplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°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 μl/well). The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.
6. Add 100 μl of diluted HRP conjugate into each well.
7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
8. Wash as detailed in 4 to 5 above.
9. Dispense 100 μl of TMB Reagent into each well.
10. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°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 5 minutes.*

**CALCULATION OF RESULTS**

1. Calculate the average absorbance values (A450) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of anti-PEG IgG in u/ml from the standard curve.
4. Multiply the derived concentration by the dilution factor to determine the actual concentration of anti-PEG IgG in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD450 values of samples fall outside the standard curve when tested at a dilution of 500, 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.

<table>
<thead>
<tr>
<th>Anti-PEG IgG (u/ml)</th>
<th>A450</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>2.635</td>
</tr>
<tr>
<td>50</td>
<td>1.351</td>
</tr>
<tr>
<td>25</td>
<td>0.660</td>
</tr>
<tr>
<td>12.5</td>
<td>0.311</td>
</tr>
<tr>
<td>6.25</td>
<td>0.182</td>
</tr>
</tbody>
</table>

**LIMITATIONS OF THE PROCEDURE**

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
3. Use only the wash buffer 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.
4. Kits are validated using shaking incubators 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.

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