ELISA for the Measurement of mPEG and mPEGylated Proteins

PLEASE READ THESE INSTRUCTIONS THOROUGHLY BEFORE USING THE KIT

INTENDED USE
This kit is for research use only. Under no circumstances should it be used for therapeutic or human diagnostic applications.

INTRODUCTION
PEGylation of therapeutic proteins by attachment of mPEG chains slows proteolytic degradation and decreases the rate of clearance from the circulatory system, thereby increasing efficacy (refs. 1 & 2). The pharmacodynamics of mPEGylated proteins are often evaluated using an assay specific for the polypeptide chain. Such an approach requires the time consuming and expensive construction of a specific ELISA. The mPEG ELISA detects the mPEG chain and is therefore suitable for assessment of the pharmacodynamics of a range of mPEGylated biologics and mPEGs.

The format of the assay is a sandwich ELISA. It uses two anti-PEG mouse monoclonal antibodies developed at Life Diagnostics, Inc. An antibody specific for the polyoxymethylene backbone of PEG (catalog# 9B5-6-25-7) is coated on the 96-well plate and used for capture, and an antibody specific for the terminal methoxy group of mPEG (catalog# 5D6-3) is conjugated to horseradish peroxidase (HRP) and used for detection.

This ELISA can be used for measurement of free mPEG (Figure 1) and mPEGylated proteins with one or more mPEG chains (Figure 2). However, it will not detect PEG chains that lack a terminal methoxy group. Sensitivity varies with mPEG chain length, therefore the mPEG or mPEGylated molecule under investigation should be used to generate a standard curve. Studies at Life Diagnostics have demonstrated that the kit recognizes mPEG chains ≥2 kDa. The standard provided with the kit is 5 kDa mPEG-amine. It is provided so that the end user can check the performance of the kit.

Figure 1. Detection of unconjugated non-branched mPEG-amines of different molecular weights using the mPEG ELISA.

Figure 2. Detection of multi-Pegylated and mono-Pegylated BSA using the mPEG ELISA. The multi-Pegylated BSA was estimated to have an average of 5-7 20 kDa mPEG chains attached per molecule of BSA.

ASSAY OVERVIEW
HRP anti-mPEG (100 μl) is first added to the microtiter wells. Then, diluted samples and standards (100 μl) are added and mixed for 1 hour on a plate shaker. After washing the wells, TMB (100 μl) is added to the wells and incubated for 20 minutes, resulting in the development of a blue color. The reaction is stopped by addition of 1N HCl (100 μl), changing the color to yellow, and absorbance at 450 nm is measured. The concentration of mPEG is derived from a standard curve.

STORAGE
Upon receipt the HRP anti-Peg vial, the mPEG standard and the anti-Peg coated 96 well plate should be placed in a -20°C freezer until use. Do not store at lower temperatures. The remainder of the kit should be stored in a refrigerator at 2-8°C. The microtiter strips 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.

MATERIALS
Materials provided with the kit:
- Anti-Peg coated 96-well Plate (12 detachable strips of 8-wells). Store at -20°C.
- HRP anti-mPEG conjugate (1 vial). Store at -20°C.
- mPEG Diluent, 50 ml
- 5 kDa mPEG-amine Standard (1 vial). Store at -20°C.
- mPEG Wash Buffer (10x stock), 60 ml
- TMB Reagent, 11 ml
- Stop Solution, 11 ml

Materials required but not provided:
- Pipettors and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Micro-plate incubator/shaker (mixing speed of ~150 rpm)
- Plate washer
- Plate reader with an optical density range of 0-4 at 450 nm
- Graphing software
PREPARATION OF KIT STANDARDS
1. Label 8 polypropylene or glass tubes as 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0 ng/ml.
2. Prepare the 20 ng/ml standard as described on the 5 kDa mPEG standard vial label.
3. Dispense 250 μl of diluent into the tubes labeled 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0 ng/ml.
4. Pipette 250 μl of the 20 ng/ml mPEG standard into the tube labeled 10 ng/ml and mix. This provides the working 10 ng/ml mPEG standard.
5. Similarly prepare the 5, 2.5, 1.25, 0.625, and 0.3125 ng/ml standards by serial dilution.

SAMPLE PREPARATION
The concentration of mPEGylated protein or mPEG in serum or plasma depends on several factors: the route of injection, the amount injected, and the time after injection at which serum or plasma is collected. Because such variables are user defined, optimum dilution must be determined empirically. A minimum 8-fold dilution of serum or plasma into the kit diluent is recommended. Higher concentrations of serum or plasma may give matrix effects, leading to inaccurate estimation of mPEG concentrations.

HRP ANTI-PEG CONJUGATE PREPARATION
Determine the volume of conjugate required (1 ml per 8-well strip) and dilute the HRP anti-mPEG conjugate stock with diluent as described on the vial label. Prepare shortly before use.

WASH SOLUTION PREPARATION
The wash solution is provided as a 10x stock. Dilute the contents of the bottle (60 ml) with 540 ml of distilled or deionized water.

ASSAY PROCEDURE
1. Secure the desired number of coated wells in the holder.
2. Add 100 μl of HRP anti-mPEG conjugate into each well.
3. Dispense 100 μl of standards and samples into the wells (standards and samples should be tested in triplicate).
4. Incubate on an orbital micro-plate shaker at 150 rpm at room temperature (25°C) for 1 hour.
5. Using a plate washer, wash the wells six times with 400 μl of wash buffer per well.
6. Dispense 100 μl of TMB reagent into each well.
7. Gently mix on an orbital micro-plate shaker at 150 rpm for 20 minutes.
8. Stop the reaction by adding 100 μl of Stop Solution to each well.
9. Gently mix until all the blue color changes to yellow.
10. Read absorbance at 450 nm with a 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, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Fit the data using an appropriate model and PC graphing software. We find that data can usually be fitted well to a two-site binding model or a second order polynomial equation.
4. Using the mean absorbance value for each sample, determine the corresponding concentration of mPEG or mPEGylated protein from the standard.
5. Multiply the concentration by the dilution factor (if applicable) to determine the actual concentration of PEGylated protein in the serum/plasma sample.
6. If the A450 values of samples fall outside the useful range of the standard curve, samples should be diluted appropriately and re-tested.

STANDARD CURVE
A representative standard curve with optical density readings at 450 nm on the Y-axis against 5 kDa mPEG-amine concentration on the X-axis is shown below.

<table>
<thead>
<tr>
<th>5 kDa mPEG-amine (ng/ml)</th>
<th>A450</th>
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<tbody>
<tr>
<td>20</td>
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<td>1.25</td>
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<tr>
<td>0.625</td>
<td>0.577</td>
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<tr>
<td>0.3125</td>
<td>0.431</td>
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<tr>
<td>0</td>
<td>0.317</td>
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</tbody>
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SPIKE/RECOVERY
Sprague Dawley rat serum was spiked with 10 ng/ml 5 kDa mPEG-amine. Recovery was 121±8% at serum dilutions of 8-32 fold.

IMPORTANT TIPS AND CONSIDERATIONS
- All reagents must be allowed to reach room temperature (18-25°C) before use.
- Add the HRP anti-PEG conjugate to the microtiter wells before adding samples and standards.
- Do not substitute user-prepared or other commercially available buffers for those provided with the kit (i.e. diluent or wash buffer). We have optimized the composition of the buffers to give a low background and low variability. Many other commercially available ELISA buffers contain PEG or PEGylated molecules and these will affect performance of the kit.
- Tween-20 is a polyoxyethylene containing detergent commonly used in ELISA dilution and wash buffers. It will interfere with this ELISA. The same is true for other
polyoxyethylene detergents. We also found that Zwitterionic detergents such as CHAPS cause interference in the assay. Detergents should therefore be excluded from sample buffers.

- The wash step is critical. A plate washer should be used with the wash buffer provided with the kit. Wash all tubing and supply bottles of the plate washer with distilled or deionized water prior to use and thoroughly prime the plate washer with wash buffer.
- Do not add azide as a preservative to serum or plasma samples. Azide is an inhibitor of HRP and will invalidate the assay.
- mPEG concentrations cannot be determined in the presence of non-methoxy PEG because the latter competes with mPEG for binding to the capture antibody.
- When preparing standards, we routinely perform serial dilutions in a blank 96-well polystyrene plate using a multipipetor. Samples are also prepared and/or dispensed into the blank 96-well plate in the layout to be used in the ELISA. This allows for quick and easy transfer of samples and standards to the ELISA plate using a multipipetor.
- If a standard other than that provided with the kit is to be used we suggest that the standard curve range be determined using 4-fold dilutions, starting with a concentration of 1000 ng/ml. Using a single 8-well strip, this allows a concentration range from 1000 ng/ml to 0.06 ng/ml to be inexpensively investigated. The useful standard curve range can then be fine-tuned.

REFERENCES

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