

# POLYETHYLENE GLYCOL (PEG) ELISA KIT

## ELISA for the Determination of mPEGylated proteins in Serum or Plasma

### INTRODUCTION

Conjugation of methoxy polyethylene glycol (mPEG) to therapeutic proteins prolongs their half-life by slowing proteolytic degradation and decreasing the rate of clearance from the circulation system (ref. 1). The pharmacodynamics of mPEGylated proteins are often evaluated using specific assays for the protein itself. That approach often requires the time consuming and expensive construction of an ELISA for the specific protein. In contrast, the mPEG ELISA

mPEG portion of the conjugate and is therefore suitable for assessment of the pharmacodynamics of a wide range of mPEG conjugated proteins.

### INTENDED USE

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

### STORAGE OF TEST KIT

Upon receipt both vials of Anti-PEG HRP Conjugate should be placed in a -80°C freezer until use. 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.

### PRINCIPLE OF THE TEST

The PEG ELISA is a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-PEG antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-PEG antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 30 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This results in PEGylated proteins being sandwiched between the immobilization and detection antibodies. 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 PEG is proportional to the optical density of the test sample. The antibodies used in this kit are of polyclonal origin and were raised against mPEGylated protein.

## MATERIALS AND COMPONENTS

### Materials provided with the kit:

- Anti-PEG antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Anti-PEG HRP Conjugate (lyophilized, 2 vials)
- Anti-PEG HRP Diluent (16 ml, pink color)
- PEG standard (lyophilized)<sup>†</sup>
- Wash Buffer (20x stock, 50 ml)
- Standard and Sample Diluent (50 ml, yellow color)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

### Materials required but not provided:

- 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 reader with an optical density range of 0.4 at 450nm
- Graph paper (PC graphing software is recommended).

## GENERAL INSTRUCTIONS

All reagents should be allowed to reach room temperature (18-25°C) before use.

### WASH SOLUTION PREPARATION

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

### PREPARATION OF STANDARDS

1. The PEG standard consists of mPEGylated bovine serum albumin (BSA)<sup>†</sup> and is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the reference standard vial label and mix gently until dissolved (**the reconstituted standard remains stable for at least 1 day at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended**).
2. Label 6 polypropylene or glass tubes as 150, 50, 16.67, 5.55, 1.85 and 0.617 ng/ml.
3. Prepare the 150 ng/ml standard as described on the PEG standard vial label.
4. Dispense 300 µl of Standard and Sample diluent into the tubes labeled 50, 16.67, 5.55, 1.85 and 0.617 ng/ml.
5. Pipette 150 µl of the 150 ng/ml PEG standard into the tube labeled 50 ng/ml and mix. This provides the working 50 ng/ml PEG-BSA standard.
6. Similarly prepare the 16.67, 5.55, 1.85 and 0.617 ng/ml standards by three-fold serial dilution.

## SAMPLE PREPARATION

The concentration of mPEGylated protein 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 it is impossible to recommend general dilution guidelines and the optimum dilution must be determined empirically. We provide Standard and Sample Diluent that should be used for dilution of serum or plasma.

## ANTI-PEG HRP CONJUGATE PREPARATION

The Anti-PEG HRP Conjugate should be prepared 5-10 minutes prior to use by adding the volume of Anti-PEG HRP Diluent listed on the vial label to one vial of lyophilized Anti-PEG HRP Conjugate. Replace the stopper securely and mix gently by inversion for ~15 seconds. One vial provides sufficient conjugate for at least 48 wells. If more than 48-wells are to be used, the second vial of Anti-PEG HRP Conjugate should be reconstituted also and the contents of both vials should be combined and mixed to ensure uniformity. Once reconstituted, the Anti-PEG HRP Conjugate is usable for 24 hours if stored at 2-8°C (it should be warmed to room temperature prior to use).

## ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100  $\mu$ l of standards and samples into the wells (we recommend that standards and samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 30 minutes.
4. Remove the incubation mixture using either a plate washer or by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 6 times with 1x wash solution. This should preferentially be performed using a plate washer (400  $\mu$ l/well). If a plate washer is not available use a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100  $\mu$ l of Anti-PEG HRP conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 30 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100  $\mu$ l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
13. Stop the reaction by adding 100  $\mu$ l of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

## CALCULATION OF RESULTS

We strongly recommend that wherever possible, PC graphing software be used to calculate results. We find that standard data generated in the PEG ELISA fit well to a "Two Site Binding (Hyperbola) Equation" ( $Y=B_{max1} \cdot X / (Kd1+X) + B_{max2} \cdot X / (Kd2+X)$ ).

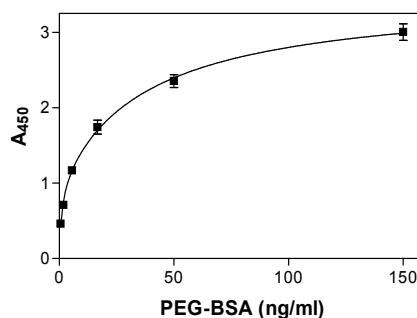
1. Calculate the average absorbance values ( $A_{450}$ ) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of PEG-BSA equivalents in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of PEG (PEG-BSA equivalents) in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. 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 PEG-BSA concentration on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns.

PEG-BSA (ng/ml)	Absorbance (450 nm)
150	3.004
50	2.354
16.67	1.742
5.55	1.168
1.85	0.710
0.617	0.463

Representative PEG Standard Curve



## 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.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

## REFERENCES

1. Webster R. et.al. PEGylated Proteins: Evaluation of their safety in the absence of definitive metabolism studies. Drug Metabolism and Disposition 35:9-16 (2007)