

# POLYETHYLENE GLYCOL BACKBONE ELISA

## Life Diagnostics, Inc., Catalog Number: PEG

### ELISA for the Measurement of PEG and PEGylated Proteins

PLEASE READ THESE INSTRUCTIONS COMPLETELY BEFORE PERFORMING THE ELISA

#### INTENDED USE

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

#### INTRODUCTION

PEGylation of biologics prolongs their half-life by slowing proteolytic degradation and decreasing the rate of clearance from the circulatory system (ref. 1). The pharmacodynamics of PEGylated 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 protein of interest. The PEG ELISA manufactured by Life Diagnostics, Inc. allows measurement of the PEG portion of the PEGylated protein and is therefore suitable for assessment of the pharmacodynamics of a range of PEGylated proteins.

#### BACKGROUND

The format of the assay is a direct competitive ELISA. BSA (bovine serum albumin) with a covalently attached single chain of methoxy-PEG is coated on the microtiter wells of a 96-well plate. Diluted samples and standards (50  $\mu$ l) containing PEGylated protein are added to the microtiter wells. Horseradish peroxidase conjugated anti-PEG (HRP anti-PEG, 50  $\mu$ l) is then added to the microtiter wells and the plate is incubated on a plate shaker for 1 hour. After washing the wells, TMB, an HRP substrate (100  $\mu$ l), is added to the wells and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped by addition of 1N HCl (100  $\mu$ l), changing the color to yellow, and absorbance at 450 nm is measured. If PEG is present in the sample, it competes with the immobilized PEG on the plate for binding to HRP anti-PEG, thereby giving lower absorbance values.

The antibody used in this ELISA is mouse monoclonal antibody (clone 1D9-6) developed by Life Diagnostics that is specific for the polyoxyethylene backbone. Studies at Life Diagnostics indicate that the kit can be used for the detection of proteins containing one or more PEG chains as well as unconjugated PEG. Sensitivity increases with PEG chain length and the degree of PEGylation (Table 1), and we therefore recommend that the PEG or PEGylated molecule under investigation be used to generate a standard curve. The standard provided with the kit is BSA with a single 20kDa mPEG chain attached. It is provided so that the end user can confirm performance of the kit.

PEG	EC <sub>50</sub> (ng/ml)
BSA-(mPEG 20 kDa) <sub>3-7</sub>	1.0 +/- 0.1
BSA-mPEG 20 kDa	3.6 +/- 0.5
BSA-PEG 10 kDa	9.4 +/- 1.1
BSA-mPEG 5 kDa	111 +/- 22
20 kDa mPEG	0.70 +/- 0.15
10 kDa PEG	0.59 +/- 0.16
5 kDa mPEG	4.65 +/- 2.32

Table 1. Characteristics of PEGylated BSA and free PEG in the High Sensitivity PEG ELISA. Please note that the concentrations of PEGylated BSA refer only to the polypeptide content and do not include mass contributed by PEG. EC<sub>50</sub> values represent mean +/- SD from 3 – 6 studies.

#### STORAGE

Upon receipt the HRP Anti-PEG vial, the PEG BSA standard and the 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.

It is important that the plate, diluent and TMB reagent equilibrate to room temperature before use.

#### MATERIALS

##### Materials provided with the kit:

- PEG Coated 96-well Microtiter Plate (12 detachable strips of 8-wells). **Store at -20°C**
- HRP anti-PEG Conjugate, 1 vial. **Store at -20°C.**
- HRP PEG Diluent, 50 ml
- PEG-BSA Standard, 1 vial.\* **Store at -20°C.**
- HRP PEG Wash Buffer (20x stock), 50 ml
- TMB Reagent, 11 ml
- Stop Solution, 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 450 nm
- Graphing software

#### PREPARATION OF KIT STANDARDS

1. Label 8 polypropylene or glass tubes as 1000, 200, 40, 8, 1.6, 0.32, 0.064 and 0 ng/ml.
2. Prepare the 1000 ng/ml standard as described on the PEG standard vial label.

\* The mPEGylated-BSA standard included in this kit was manufactured at Life Diagnostics, Inc. A single 20 kDa mPEG chain is attached per BSA molecule. The concentration indicated on the vial refers only to the BSA protein concentration.

3. Dispense 200  $\mu\text{l}$  of diluent into the tubes labeled 200, 40, 8, 1.6, 0.32, 0.064 and 0 ng/ml.
4. Pipette 50  $\mu\text{l}$  of the 1000 ng/ml PEG standard into the tube labeled 200 ng/ml and mix. This provides the working 200 ng/ml PEG-BSA standard.
5. Similarly prepare 40, 8, 1.6, 0.32, and 0.064 ng/ml standards by five-fold serial dilution.

### SAMPLE PREPARATION

The concentration of PEGylated 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, optimum dilution must be determined empirically.

### HRP ANTI-PEG CONJUGATE PREPARATION

Determine the volume of conjugate required (0.5 ml per 8-well strip) and dilute the HRP anti-PEG conjugate stock with diluent as described on the vial label. Prepare shortly 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.

### ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 50  $\mu\text{l}$  of standards and samples into the wells (we recommend that standards and samples be tested in triplicate).
3. Add 50  $\mu\text{l}$  of HRP anti-PEG conjugate into each well.
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  $\mu\text{l}$  of wash buffer per well.
6. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
7. Dispense 100  $\mu\text{l}$  of TMB reagent into each well.
8. Gently mix on an orbital micro-plate shaker at 150 rpm for 20 minutes.
9. Stop the reaction by adding 100  $\mu\text{l}$  of Stop Solution to each well.
10. Gently mix until all the blue color changes to yellow.
11. Read absorbance at 450 nm with a plate reader within 5 minutes.

### CALCULATION OF RESULTS

PC graphing software should be used to calculate results.

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 the  $\log_{10}$  of 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 to a sigmoidal dose response (variable slope) model. The upper limit of the curve may be fixed at the value defined by the 0 ng/ml "standard" and the lower limit of the curve may be fixed at the value of the 1000 ng/ml kit standard.
4. Using the mean absorbance value for each sample, determine the corresponding  $\log_{10}$  concentration of PEGylated protein from the standard curve and derive the concentration in ng/ml by calculating the anti- $\log_{10}$ .
5. We strongly recommend that only absorbance values of samples falling within the middle 50% region of the standard curve be used to determine PEG concentrations. For example,

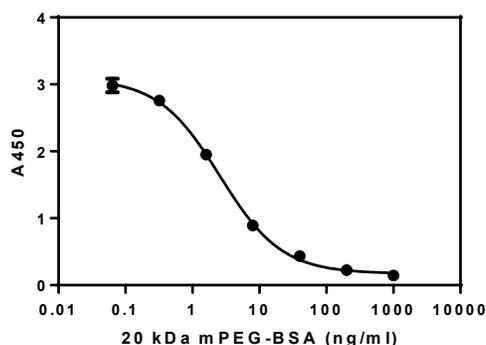
if the absorbance values of the low (zero ng/ml) and high standards are 1.6 and 0.1, only use sample absorbance values falling within the 1.225 and 0.475 range.

6. Multiply the derived concentration by the dilution factor to determine the actual concentration of PEGylated protein in the serum/plasma sample.
7. If the  $A_{450}$  values of samples fall outside the useful range of the standard curve, samples should be diluted appropriately and re-tested.

### REPRESENTATIVE STANDARD CURVE

A representative standard curve with optical density readings at 450 nm on the Y axis against BSA-mPEG 20 kDa concentration on the X axis is shown below. Please note that the X-axis is a  $\log_{10}$  scale.

BSA-mPEG 20 kDa (ng/ml)	$A_{450}$
1000	0.145
200	0.226
40	0.436
8	0.890
1.6	1.951
0.32	2.757
0.064	2.983
0	3.212



### SPIKE/RECOVERY DATA

Sprague Dawley rat serum was spiked with the indicated concentrations of BSA-mPEG 20 kDa and recovery estimated using PEG ELISA.

Spike (ng/ml)	Recovered (ng/ml)	n
1000	1108 +/- 225	3
100	101 +/- 31.6	3
10	38.7 +/- 4.7	3
0	30	1

We find that normal rat serum contains measurable levels of PEG, possibly from dietary sources.

### IMPORTANT TIPS

- All reagents must be allowed to reach room temperature (25°C) before use.
- **ALWAYS** add samples and standards to the microtiter wells before adding the HRP anti-PEG conjugate.
- Do not substitute user-prepared buffers for those provided with the kit (i.e. diluent or wash buffer). Many commercially available materials contain PEG or PEGylated molecules, and

this will affect performance of the kit. For this reason, care should be taken when selecting other components used in studies of PEGylated proteins.

- 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.
- Do not add azide as a preservative to serum or plasma samples. Azide is an inhibitor of HRP and will invalidate the assay.
- We strongly recommend that a plate washer be used to wash the microtiter wells. Only use the wash buffer provided with the kit. Wash all tubing and vessels of the plate washer with distilled or deionized water prior to use and thoroughly prime the plate washer with wash buffer.
- When preparing standards, we routinely perform appropriate serial dilutions in a blank 96-well polystyrene plate using a multipipettor. 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 multipipettor.
- If a standard other than that provided with the kit is to be used, we suggest that the standard curve range be determined using 10-fold dilutions, starting with a concentration of 10 µg/ml. Using a single 8-well strip, this allows a concentration range from 0.1 ng/ml to 10 µg/ml to be inexpensively investigated. The useful standard curve range can then be fine-tuned.

#### **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)

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For technical assistance please email us at  
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