

POLYETHYLENE GLYCOL (PEG) ELISA KIT

Life Diagnostics, Inc., Catalog Number: P-0002

ELISA for the Measurement of PEG and PEGylated Proteins

INTRODUCTION

Conjugation of polyethylene glycol (PEG) 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 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 with a single 10 kDa chain of non-methoxy PEG is coated onto the microtiter wells of a 96-well plate. During the assay, diluted samples and standards (100 μ l) containing PEGylated protein are added to the microtiter wells. Alkaline phosphatase conjugated anti-PEG (AP anti-PEG, 100 μ l) is then added to the microtiter wells and the plate is incubated on a plate shaker for 2 hours. After washing the wells, 200 μ l of para-nitrophenyl phosphate (pNPP) substrate solution is added and the plate incubated on a plate shaker for 1.5 hours. Absorbance at 405 nm is then measured using a plate reader. If PEG is present in the sample, it competes with the immobilized PEG on the plate for binding to AP anti-PEG. Higher PEG concentrations in the sample result in decreased binding of AP anti-PEG to the immobilized PEG and thereby give lower absorbance values. The antibody used in this ELISA is a rabbit polyclonal antibody that was affinity purified on agarose to which non-methoxy PEG had been attached. The ELISA is thus specific for the polyoxyethylene chain.

Studies at Life Diagnostics indicate that the kit can be used for the detection of proteins containing one or more PEG chains per protein molecule (Fig. 1. and Table 1). It can also detect free PEG (Table 1). Sensitivity increases with the degree of PEGylation and with PEG chain length (Table 1). The EC_{50} and slope of the competition curve varies with the extent of PEGylation (number of PEG chains per protein molecule) and with PEG chain length. For these reasons, we recommend that the investigator generate a standard curve using the PEGylated protein under investigation. The standard provided with the kit consists of pure BSA with 3-7 20kDa mPEG chains attached per molecule. It is included in the kit so that the end user can confirm performance of the kit. It may, or may not, provide a suitable standard for other PEGylated proteins. BSA to which a single 20 kDa mPEG chain is attached is available from Life Diagnostics, Inc., for use as an alternative standard (Cat. No., PBSA-01).

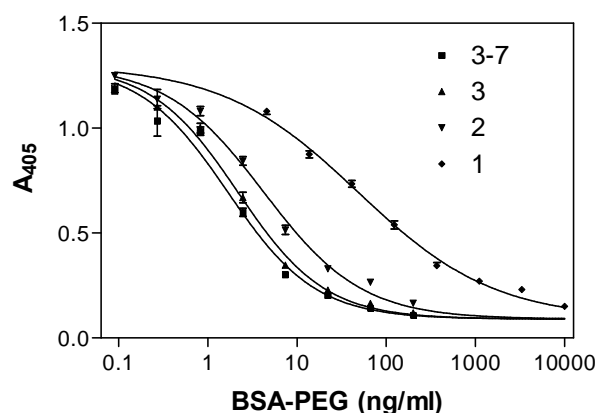


Fig.1. Effect of the extent of PEGylation. BSA with 1, 2, 3 or 3-7 20kDa mPEG chains per molecule were tested at the concentrations indicated.

PEG	EC_{50} (ng/ml)	Slope
BSA-(mPEG 20 kDa) ₃₋₇ , Kit Standard	2.8	-0.99
BSA-(mPEG 20 kDa) ₃	4.7	-1.19
BSA-(mPEG 20 kDa) ₂	6.5	-0.99
BSA-(mPEG 20 kDa) ₁	69	-0.53
BSA-(mPEG 5 kDa) ₄	104	-0.47
BSA-(mPEG 5 kDa) ₃	151	-0.43
BSA-(mPEG 5 kDa) ₂	451	-0.40
BSA-(mPEG 5 kDa) ₁	-	-
2 kDa mPEG	66864	-0.55
5 kDa mPEG	759	-0.59
10 kDa PEG	197	-0.61
20 kDa mPEG	38	-0.62
10 kDa 4-Arm PEG (4 x 2.5 kDa)	1951	-0.60
20 kDa 4-Arm PEG (4 x 5 kDa)	33	-0.51

Table 1. Characteristics of PEGylated BSA and free PEG in the PEG ELISA. Please note that the concentrations of PEGylated BSA refer only to the polypeptide content and do not include mass contributed by PEG.

INTENDED USE

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

STORAGE

Upon receipt the AP 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.

MATERIALS

Materials provided with the kit:

- PEG coated 96-well microtiter plate (provided as 12 detachable strips of 8-wells). **Store at -20°C**
- AP Anti-PEG Conjugate, 20 µl (1 vial). **Store at -20°C**. Do not store at lower temperatures.
- PEG Diluent, 50 ml
- PEG-BSA standard, 20 µl (1 vial)¹. **Store at -20°C**. Do not store at lower temperatures.
- Wash Buffer (10x stock), 50 ml
- pNPP substrate tablets, 6 tablets
- pNPP Buffer, 30 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 0-2 at 405nm
- Graph paper (PC graphing software is recommended).

PREPARATION OF KIT STANDARDS

1. The PEG-BSA standard consists of mPEGylated bovine serum albumin (BSA)¹ and is provided as a 50% glycerol stock.
2. Label 8 polypropylene or glass tubes as 200, 66.7, 22.2, 7.4, 2.47, 0.82, 0.27 and 0.09 ng/ml.
3. Prepare the 200 ng/ml standard as described on the PEG standard vial label.
4. Dispense 200 µl of diluent into the tubes labeled 66.7, 22.2, 7.4, 2.47, 0.82, 0.27 and 0.09 ng/ml.
5. Pipette 100 µl of the 200 ng/ml PEG standard into the tube labeled 66.7 ng/ml and mix. This provides the working 66.7 ng/ml PEG-BSA standard.
6. Similarly prepare the 22.2, 7.4, 2.47, 0.82, 0.27 and 0.09 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.

AP ANTI-PEG CONJUGATE PREPARATION

Determine the volume of conjugate required and dilute the AP anti-PEG conjugate stock with diluent as described on the stock vial label. Prepare shortly before use.

WASH SOLUTION PREPARATION

The wash solution is provided as a 10x stock. Dilute the contents of the bottle (50 ml) with 450 ml of distilled or deionized water.

pNPP SUBSTRATE PREPARATION

Determine the approximate volume of substrate solution required and round up to the nearest 5 ml. Assume that 0.20 ml are required per well. For example, if 2 x 8-well strips are to be used in the ELISA, you will need a total of 16 x 0.2 ml = 3.2 ml of pNPP substrate solution. That rounds-up to 5 ml. For every 5 ml of substrate solution required add one pNPP tablet per 5 ml of pNPP buffer and mix gently for 5-10 min in order to dissolve the tablet(s). It is important to prepare the pNPP substrate solution prior to the wash step in order to avoid the possibility of the washed wells drying out.

IMPORTANT TIPS

- All reagents must be allowed to reach room temperature (18-25°C) before use.
- **ALWAYS** add sample to the microtiter wells before adding the AP 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 also, care should be taken when selecting all components used in studies of PEGylated proteins.
- We strongly recommend that a plate washer be used to wash the microtiter wells. Please use only the wash buffer provided with the kit. Wash all tubing and vessels of the plate washer with distilled or deionized water prior to use then 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.
- It is important to prepare the pNPP substrate solution before initiating the wash step. It takes 5-10 min for the pNPP tablets to dissolve and the plate must not be allowed to dry during this time.
- 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 100 µg/ml. Using a single 8-well strip, this allows a concentration range from 0.01 ng/ml to 100 µg/ml to be inexpensively investigated. The useful standard curve range can then be fine-tuned.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards and samples into the wells (we recommend that standards and samples be tested in duplicate).
3. Add 100 µl of AP Anti-PEG conjugate into each well.

¹ The mPEGylated-BSA standard used in this kit was manufactured at Life Diagnostics, Inc. SDS PAGE analysis indicates that 3-7 20 kDa mPEG chains are attached per BSA molecule. It is referred to as BSA-(mPEG 20 kDa)₃₋₇.

4. Incubate on an orbital micro-plate shaker at 150 rpm at room temperature (25°C) for 2 hours².
5. Using a plate washer, wash the wells six times with 400 µl of wash buffer per well.
6. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
7. Dispense 200 µl of pNPP substrate solution into each well.
8. Gently mix on an orbital micro-plate shaker at 150 rpm at room temperature for 1.5 hours.
9. Read the optical density at 405 nm with a microtiter plate reader.

BSA-(mPEG 20 kDa) ₃₋₇ (ng/ml)	A ₄₀₅
200.00	0.100
66.67	0.138
22.22	0.172
7.41	0.298
2.47	0.548
0.82	0.829
0.27	0.919
0.09	1.135

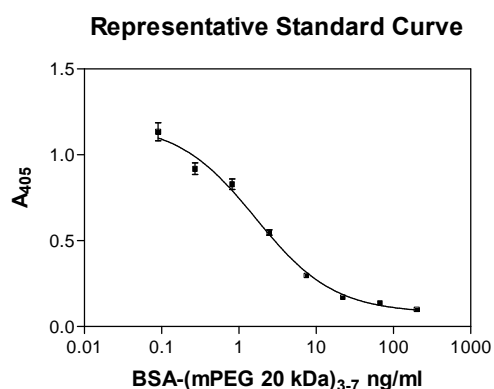
CALCULATION OF RESULTS

PC graphing software should be used to calculate results.

1. Calculate the average absorbance values (A₄₀₅) 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 log₁₀ of its concentration in ng/ml, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using PC graphing software, fit the data to a sigmoidal dose response (variable slope) model.
4. Using the mean absorbance value for each sample, determine the corresponding log₁₀ concentration of PEGylated protein from the standard curve and derive the concentration in ng/ml by calculating the anti-log₁₀.
5. Multiply the derived concentration by the dilution factor to determine the actual concentration of PEGylated protein in the serum/plasma sample.
6. If the A₄₀₅ values of samples fall outside the standard curve Samples should be diluted appropriately and re-tested.

REPRESENTATIVE STANDARD CURVE

A representative standard curve with optical density readings at 405nm on the Y axis against BSA-(mPEG 20 kDa)₃₋₇ concentration on the X axis is shown below. Please note that the X-axis is a log₁₀ scale.



² The assay was validated at 25°C using a temperature controlled shaker-incubator. Incubation at lower temperatures will result in decreased absorbance values.

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)

Life Diagnostics, Inc.

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Mono PEGylated BSA ***(PEGylated with 20kDa mPEG maleimide)***

Catalog No.	PBSA-01
Analysis	SDS PAGE & HPLC
Purity	≥90%
Concentration	100 - 500 µg/ml (Assuming an extinction coefficient of 0.632 at 280 nm for a 1 mg/ml solution and path length of 1 cm).
Buffer	150 mM NaCl, 10 sodium phosphate, 0.1% NaN ₃ , (pH 7.2)
Storage	-20°C (Safe for shipping at 2-8°C)
Comments	<p>Prepared by conjugation of mPEG-maleimide (20 kDa) to native BSA followed by gel-filtration chromatography to remove free PEG and unconjugated BSA.</p> <p>BSA contains a single maleimide-reactive thiol at Cys-34. Reaction of BSA with mPEG-maleimide therefore results in the covalent attachment a single PEG molecule per molecule of BSA.</p>

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Rabbit Anti-Polyethylene Glycol (PEG)

Catalog No.	PEGPAB-01
Description	Rabbit anti-PEG IgG. Purified by affinity chromatography on non-methoxy PEG agarose and ion-exchange chromatography.
Purity	>90% by SDS PAGE
Protein	0.5 - 5 mg/ml (E1% at 280 nm = 14.0)
Applications	ELISA, Western Blot
Buffer	10 mM Sodium Phosphate, 150 mM NaCl, pH 7.2, 0.10% NaN ₃
Storage	2-8°C short term, -20°C or lower long term (avoid repeated freeze-thaws)
Immunogen	mPEGylated ovalbumin

Comments: This antibody recognizes the PEG polymer chain, not the terminal methoxy group. It will recognize both methoxy-PEG and non methoxy-PEG. It recognizes PEG's with a molecular weight of 550 or greater in direct ELISA and western blot assays. Studies at LDI indicate that it pairs well, as a detection antibody, when used with monoclonals 3F12-1 and 10E3-1-4 as coating antibodies. It can be efficiently conjugated to alkaline phosphatase using glutaraldehyde.

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Polyethylene Glycol (PEG) Monoclonal Clone 10E3-1-4

Catalog No.	10E3-1-4
Description	Mouse anti-PEG IgG1k Purified by Protein-G affinity chromatography from serum-free culture medium
KD	3.7 x 10 ⁻⁸ M (Determined using an Octet QK™ in a buffer of 1M NaCl, 5 mM Tris.Cl, pH 7.4, 0.1% NaN ₃ , 7% w/v BSA, using biotin-20 kDa mPEG as ligand)
Purity	>95% by SDS PAGE
Protein	1 - 5 mg/ml (E1% at 280 nm = 14.0)
Applications	Direct & Sandwich ELISA, Western Blot
Buffer	10 mM Sodium Phosphate, 150 mM NaCl, pH 7.2, 0.10% NaN ₃
Storage	2-8°C
Immunogen	mPEG-Ovalbumin

Comments: This antibody recognizes the PEG polymer chain, not the terminal methoxy group. It will recognize both methoxy-PEG and non-methoxy PEG. It recognizes PEG's with a molecular weight of 550 or greater in indirect ELISA. Studies at LDI indicate it can be used as coating antibody in sandwich ELISA when paired with monoclonal 10B4-2 as detection antibody.

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Polyethylene Glycol (PEG) Monoclonal Clone 10B4-2

Catalog No.	10B4-2
Description	Mouse anti-PEG IgG1k Purified by Protein-G affinity chromatography from serum-free culture medium
KD	2.28 x 10 ⁻⁸ M (Determined using an Octet QK™ in a buffer of 1M NaCl, 5 mM Tris.Cl, pH 7.4, 0.1% NaN ₃ , 7% w/v BSA, using biotin-20 kDa mPEG as ligand)
Purity	>95% by SDS PAGE
Protein	1 - 5 mg/ml (E1% at 280 nm = 14.0)
Applications	Direct, indirect & sandwich ELISA, Western Blot
Buffer	10 mM Sodium Phosphate, 150 mM NaCl, pH 7.2, 0.10% NaN ₃
Storage	2-8°C
Immunogen	mPEG-Ovalbumin

Comments: This antibody recognizes the PEG polymer chain, not the terminal methoxy group. It will recognize both methoxy-PEG and non-methoxy PEG. It recognizes PEG's with a molecular weight of 550 or greater in direct ELISA. Studies at LDI indicate it can be used in indirect ELISA and as conjugate antibody in sandwich ELISA. It can be coupled to alkaline phosphatase using glutaraldehyde and pairs with monoclonals 3F12-1 and 10E3-1-4 as coating antibodies.

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Polyethylene Glycol (PEG) Monoclonal Clone 3F12-1

Catalog No.	3F12-1
Description	Mouse anti-PEG IgG1k Purified by Protein-G affinity chromatography from serum-free culture medium
KD	1.84 x 10 ⁻⁸ M (Determined using an Octet QK™ in a buffer of 1M NaCl, 5 mM Tris.Cl, pH 7.4, 0.1% NaN ₃ , 7% w/v BSA, using biotin-20 kDa mPEG as ligand)
Purity	>95% by SDS PAGE
Protein	1 - 5 mg/ml (E1% at 280 nm = 14.0)
Applications	Indirect & Sandwich ELISA, Western Blot
Buffer	10 mM Sodium Phosphate, 150 mM NaCl, pH 7.2, 0.10% NaN ₃
Storage	2-8°C
Immunogen	mPEG-Ovalbumin

Comments: This antibody recognizes the PEG polymer chain, not the terminal methoxy group. It will recognize both methoxy-PEG and non-methoxy PEG. It recognizes PEG's with a molecular weight of 550 or greater in indirect ELISA. Studies at LDI indicate it can be used in indirect ELISA and as a coating antibody in sandwich ELISA.

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Polyethylene Glycol (PEG) Monoclonal Clone 1D9-6

Catalog No.	1D9-6
Description	Mouse anti-PEG IgG1k Purified by Protein-G affinity chromatography from serum-free culture medium
KD	2.9x 10 ⁻⁹ M (Determined using an Octet QK™ in a buffer of 1M NaCl, 5 mM Tris.Cl, pH 7.4, 0.1% NaN ₃ , 7% w/v BSA, using biotin-20 kDa mPEG as ligand)
Purity	>95% by SDS PAGE
Protein	0.5 - 5 mg/ml (E1% at 280 nm = 14.0)
Applications	Indirect ELISA, Western Blot
Buffer	10 mM Sodium Phosphate, 150 mM NaCl, pH 7.2, 0.10% NaN ₃
Storage	2-8°C
Immunogen	mPEG-Ovalbumin

Comments: This antibody recognizes the PEG polymer chain, not the terminal methoxy group. It will recognize both methoxy-PEG and non methoxy-PEG. It recognizes PEG's with a molecular weight of 550 or greater in indirect ELISA.

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Mono PEGylated BSA ***(PEGylated with 5 kDa mPEG maleimide)***

Catalog No.	PBSA-02
Analysis	SDS PAGE & HPLC
Purity	≥90%
Concentration	200 - 1000 µg/ml (Assuming an extinction coefficient of 0.632 at 280 nm for a 1 mg/ml solution and path length of 1 cm).
Buffer	150 mM NaCl, 10 sodium phosphate, 0.1% NaN ₃ , (pH 7.2)
Storage	-20°C (Safe for shipping at 2-8°C)
Comments	<p>Prepared by conjugation of mPEG-maleimide (5 kDa) to native BSA followed by gel-filtration chromatography to remove free PEG and unconjugated BSA.</p> <p>BSA contains a single maleimide-reactive thiol at Cys-34. Reaction of BSA with mPEG-maleimide therefore results in the covalent attachment a single PEG molecule per molecule of BSA.</p>

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