Sandwich ELISA for detection of mPEGylated proteins kit (MAK-0009)

This product is for research use only and is not intended for diagnostic use.

PRODUCT INFORMATION

Cat No.	MAK-0009
Storage	Sandwich ELISA
Principle	This kit is for research use only. Under no circumstances should it be used for therapeutic or human diagnostic applications.
Intended Use	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 . 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 Mabioway Co., Ltd. An antibody specific for the polyoxyethylene backbone of PEG is coated on the 96-well plate and used for capture, and an antibody specific for the terminal methoxy group of mPEG 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 . 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 Mabioway 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.

Introduction

The assay uses two different monoclonal antibodies that recognize the PEG backbone. One is coated on wells of the microtiter plate and is used for capture; the other is conjugated to horseradish peroxidase (HRP) and is used for detection. Serum samples are diluted at least 10-fold in the provided dilution buffer and incubated alongside reference standards1 in the microtiter wells for 45-minutes. The wells are subsequently washed. HRP conjugate is added and incubated for 45 minutes. If present, PEG molecules are sandwiched between the capture and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentrations of mPEG in the samples are derived from a standard curve.

Principle of the assay

PEGylation of biologics prolongs their half-life by slowing proteolytic degradation and decreasing the rate of clearance from the circulatory system. 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 Mabioway Co., Ltd. allows measurement of the PEG portion of the PEGylated protein and is therefore suitable for assessment of the pharmacodynamics of a range of mPEGylated proteins.

Storage Note

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 4°C.

Materials

Materials provided with the kit:

- Anti-PEG coated plate (12 x 8-well strips)
- HRP anti-mPEG conjugate (1 vial)
- mPEG Diluent: MPEGD50-1, 50 ml
- 5 kDa mPEG-amine Standard (1 vial)
- mPEG Wash Buffer: MPEGW60-10, 60 ml
- TMB: TMB11-1, 11 ml
- Stop Solution: SS11-1, 11 ml

General instructions

Please fully read and thoroughly understand the instructions before using the kit.

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

	2.The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. 3.Use only the wash solution and dilution buffers provided with the kit. They are specially formulated for measurement of PEG. 4.Kits are validated using plate shakers set at 150 rpm and 25°C. Performance of the assay at lower temperatures and/or mixing speeds will result in lower absorbance values. 5.Optimal results are achieved if at each step, reagents are pipetted into the wells of the microtiter plate within 5 minutes.
Diluent	The diluent (PEGBC50-1) is formulated for measurement PEG. It is supplied ready to use. DO NOT substitute other buffers.
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	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	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.
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.

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.

Important Tips

• All reagents must be allowed to reach room temperature (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 multipipettor. Samples are also prepared and/or dispensed into the blank 96well 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 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.

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 retested.