METHOXY-PEG SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: MPEG-SP

IMPORTANT DISCLAIMER

This kit is intended for research use only. Under no circumstances should it be used for human diagnostic purposes.

INTRODUCTION

The attachment of methoxy-PEG (mPEG) chains to therapeutic proteins increases their efficacy by slowing proteolytic degradation and decreasing the rate of clearance from the circulatory system (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 immunoassay. The mPEG SPARCL™ assay detects the mPEG chain and is therefore suitable for assessment of the pharmacodynamics of a range of mPEGylated biologics and unconjugated mPEGs.

PRINCIPLE OF THE ASSAY

The mPEG SPARCL™[1] (Spatial Proximity Analyte Reagent Capture Luminescence, ref 3) assay uses two PEG specific monoclonal antibodies developed at Life Diagnostics. A terminal methoxy group specific antibody (5D6-3) is conjugated to horseradish peroxidase (HRP) and a PEG backbone specific antibody (9B5-6-25-7) is conjugated to acridan, a chemiluminescent substrate. When HRP and acridan conjugated antibodies bind to mPEG they are brought into close proximity. With the addition of hydrogen peroxide, HRP catalyzes oxidation of proximal acridan molecules causing a flash of chemiluminescence. Acridan conjugated antibodies distant from HRP produce no signal. This principle allows the development of a homogeneous assay that allows rapid determination of mPEG concentrations.

One anti-methoxy group HRP-conjugated antibody (5D6-3) can bind per mPEG chain but multiple anti-backbone acridan-conjugated antibodies (9B5-6-25-7) can bind, depending on the chain length[2]. The ratio of bound acridan and HRP antibodies influences the luminescence signal and assay sensitivity (Figures 1 & 2, Table 1).

The HRP and acridan conjugated antibodies provided with the kit are mixed with standards and diluted samples in wells of the 96-well SPARCL™ plate provided with the kit[3]. After incubation for 30 minutes on a shaker at 25°C and 150 rpm, the plate is placed into a luminometer. Trigger solution containing hydrogen peroxide is injected into each well and luminescence is immediately measured. The concentration of mPEG is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-mPEG HRP stock **Store ≤ -70°C**
- Anti-PEG acridan stock **Store ≤ -70°C**
- 10 kDa mPEG-amine stock **Store ≤ -70°C**
- Diluent: PEGD50-1, 2 x 50 ml
- Trigger solution: TS7-1, 7 ml
- White SPARCL™ plate (12 x 8-well)
- Clear untreated 96-well plate

Materials required but not provided:

- Precision pipettes and tips
- Polypropylene tubes
- Vortex mixer
- Plate incubator/shaker
- Luminometer capable of simultaneous injection & measurement
- Curve fitting software

STORAGE

Store the HRP conjugate, acridan conjugate and mPEG stock at or below -70°C (they may be stored at -20°C for one week). The remainder of the kit should be stored at 2-8°C. The SPARCL™ plate should be kept in a sealed bag with desiccant and antioxidant. The kit will remain stable for at least six months from the date of purchase, provided that the components are stored appropriately.

GENERAL INSTRUCTIONS

1. Please take the time to completely read all instructions before starting your assay. Contact us if you need clarification.
2. All reagents used in the assay should be allowed to reach room temperature (25°C) before use.
3. It is important that standards and samples be added to the SPARCL™ plate quickly. If testing large numbers of samples, rather than pipetting standards and samples directly into the white SPARCL™ plate using a single channel pipettor, we recommend the following. First, pipette an excess volume of standards and samples into appropriate wells of the clear 96-well plate. Then use an 8- or 12-channel multipipettor to quickly and efficiently transfer 50 µl aliquots to the appropriate wells of the white SPARCL™ plate. The wells of the clear plate hold a maximum volume of 300 µl.
4. Follow the sequence of events below when running the assay.

Prime and program the Luminometer
⇩
Prepare standards and diluted samples
⇩
Prepare HRP + Acridan conjugate mix
⇩
Add HRP + Acridan conjugate mix to the wells (25 µl)
⇩
Add standards and samples to the wells (50 µl)
⇩
Incubate plate at 150 rpm/25°C for 30 min
⇩
Measure luminescence after injection of Trigger (37.5 µl)

PREPARATION OF CONTROL STANDARDS

Each user must construct a standard curve using the mPEGylated molecule that they are studying (see next section). We provide a stock of 10 kDa mPEG-amine so that it can be used to demonstrate that the kit works appropriately.

[1] The SPARCL technology was developed by Lumigen Corp.

[2] The minimum chain length required for 9B5-6-25-7 binding is approximately 20 oxyethylene units long (~ 1 kDa).

[3] The plate provided with the kit has been treated with a reagent that reduces background chemiluminescence. Untreated plates cannot be used.

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1. Label 8 polypropylene tubes as 100, 33.33, 11.11, 3.70, 1.24, 0.412, 0.137 and 0.046 ng/ml.
2. Into the tube labeled 100 ng/ml, pipette the volume of diluent detailed on the 10 kDa mPEG-amine stock vial label. Then add the indicated volume of stock and mix gently. This provides the 100 ng/ml standard.
3. Dispense 150 µl of diluent into the tubes labeled 33.33, 11.11, 3.70, 1.24, 0.412, 0.137 and 0.046 ng/ml.
4. Pipette 75 µl of the 100 ng/ml mPEG standard into the tube labeled 33.33 ng/ml and mix. This provides the 33.33 ng/ml mPEG standard.
5. Similarly prepare the remaining standards by 3-fold serial dilution.

TIPS FOR PREPARATION OF STANDARDS

As discussed in the "principle of the assay" section, the sensitivity of the assay depends on mPEG chain length and extent of PEGylation (Figures 1 & 2, Table 1). For these reasons, it is essential that the mPEG or mPEGylated protein that is being studied be used to construct a standard curve for quantification of unknowns. We recommend that users initially prepare a standard of 10,000 ng/ml. Standards of 1667, 278, 46.3, 7.7, 1.29, 0.21 and 0.036 should then be prepared by six-fold serial dilution (40 µl into 200 µl). These standards should then be evaluated using a single eight-well strip. Based on the results from such an experiment, optimum standard concentrations can be defined.

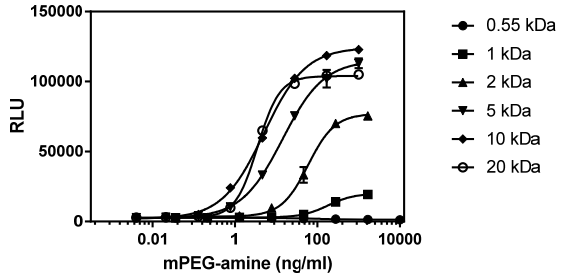


Figure 1. Standard curves obtained with mPEG-amines of varying molecular weights.

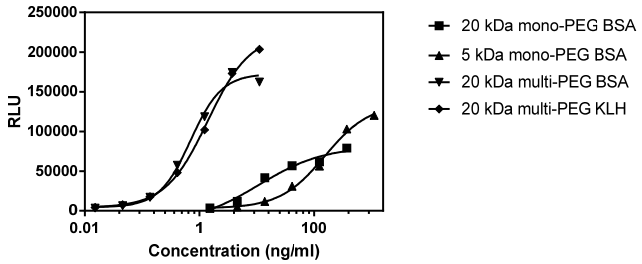


Figure 2. Standard curves obtained with mono- and multi-PEGylated proteins.

mPEG	IC_{50} (ng/ml)
0.55 kDa mPEG-amine	-
1 kDa mPEG-amine	180
2 kDa mPEG-amine	59
5 kDa mPEG-amine	14.2
10 kDa mPEG-amine	5.1
20 kDa mPEG-amine	3.5
5 kDa mono-mPEG-BSA	154
20 kDa mono-mPEG-BSA	11.0
20 kDa multi-mPEG-BSA	0.7
20 kDa multi-mPEG-KLH	1.4

Table 1. IC_{50} values of mPEG-amines and mPEGylated proteins.

SAMPLE PREPARATION

The diluent provided with the kit was designed for dilution of serum and heparinized plasma samples (EDTA or citrate plasma should not be used). The user must determine the optimal dilution(s) of samples so that potential matrix or prozone effects are eliminated.
In studies at Life Diagnostics, we spiked 10 kDa mPEG amine into cynomolgus monkey serum at concentrations of 100 and 10 ng/ml. Matrix effects could be avoided and expected recoveries obtained at serum dilutions of 16-fold or greater.

CONJUGATE MIX PREPARATION

Instructions for preparation of the conjugate mix are detailed on the box that contains the HRP and acridan conjugates. Prepare the mix shortly before it is required using the diluent provided with the kit.

LUMINOMETER SETUP

1. The luminometer must be capable of injection and simultaneous measurement of luminescence without any delay.
2. Prime the luminometer injection port with 1 ml of trigger solution.
3. Place the injection needle into the injection port as needed for BMG luminometers.
4. Program the luminometer to inject 37.5 µl of trigger solution per well and to measure from time zero for 1 second (50 x 0.02 second intervals).
5. Define the format of the assay using the luminometer software.
6. Because the white SPARCL™ plate is provided as a 12 x 8-well strips, allowing use of fewer than 96-wells, make sure that the luminometer is programmed to inject trigger solution only into the wells being used.
7. We use a BMG LUMIstar Omega set at a gain of 3600. Optimal gain should be determined by the end user.
8. There are a number of manufacturers of luminometers that are equipped to run a SPARCL™ assay. Please contact Life Diagnostics or Lumigen (www.lumigen.com) to discuss your luminometer.

PROCEDURE

1. Before starting the assay ensure that the luminometer is primed with trigger solution and that the injection needle is positioned in the injection port.
2. Secure the desired number of SPARCL™ 8-well strips in the holder. Immediately seal unused strips in the resealable bag with desiccant and antioxidant. Store unused strips at 2-8°C.
3. Aliquot 25.0 µl of conjugate mix into each well.
4. Dispense 50.0 µl of user-prepared standards, control standards (if desired) and diluted samples into the wells (we recommend that standards and samples be tested in duplicate).
5. Incubate on an orbital micro-plate shaker at 150 rpm and 25°C for 30 minutes.
6. After the 30-minute incubation, place the plate in the luminometer and measure luminescence after injection of trigger solution (37.5 µl).
7. Remove the plate from the luminometer and discard the used strips. Keep the plate frame if future use is intended.

CALCULATION OF RESULTS

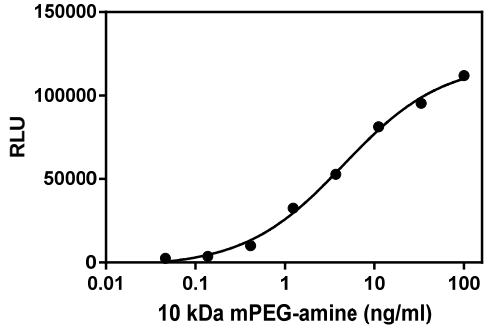
1. Before calculating results, review the raw data. If artefacts (RLU spikes) are apparent immediately after injection of trigger solution, eliminate that portion of the luminescence profile from analysis for all wells. We routinely use the sum of RLU values from a 100-980 ms data collection window.

2. Determine the sum of RLU values within the data collection window for the standards and samples.
3. Using curve fitting software, construct a standard curve by plotting the sum of RLU values for the standards versus the $\log_{10}$ of the concentration and fit to a sigmoidal, 4PL model.
4. Derive the concentration of mPEG or mPEGylated protein in the samples from the standard curve (remember to derive the concentration from the antilog).
5. Multiply the derived concentration by the dilution factor to determine the concentration in the original sample.
6. If the sum of RLU values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL CONTROL STANDARD CURVE

A typical control standard curve with sum of RLU plotted on the Y-axis versus $\log_{10}$ 10 kDa mPEG-amine concentrations on the X-axis is shown below. This curve is for illustration only and should not be used to calculate unknowns. An appropriate standard curve must be run in each experiment. The IC_{50} value of for 10 kDa mPEG-amine should be approximately 5 ng/ml.

10 kDa mPEG (ng/ml)	RLU
100	111943
33.33	95340
11.11	81320
3.70	52804
1.24	32554
0.412	10004
0.137	3670
0.046	2533



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