PEG SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: PEG-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 polyethylene glycol (PEG) 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 PEGylated 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 PEG SPARCL™ assay detects the PEG chain and is therefore suitable for assessment of the pharmacodynamics of a range of PEGylated biologics and unconjugated PEGs. Whereas our methoxy-PEG (mPEG) SPARCL kit™ (MPEG-SP) detects only mPEG, this kit detects all linear PEG chains >5 kDa.[1]

PRINCIPLE OF THE ASSAY

The PEG SPARCL™[2] (Spatial Proximity Analyte Reagent Capture Luminescence, ref 3) assay uses a monoclonal antibody (1D9-6) developed at Life Diagnostics that binds to the PEG backbone. Two 1D9-6 conjugates are used; horseradish peroxidase (HRP) and acridan, a chemiluminescent substrate. When HRP and acridan conjugated antibodies bind to PEG 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 PEG concentrations.

In studies at Life Diagnostics, we estimated that 1D9-6 binds to a portion of the PEG backbone approximately 20 oxyethylene units long (~1 kDa). Higher molecular weight PEG chains and proteins PEGylated at multiple sites bind multiple acridan and HRP conjugates, influencing the luminescence signal and assay sensitivity (Figures 1 & 2, Table 1).

The HRP and acridan conjugates 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 PEG is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-PEG HRP stock **Store ≤ -70°C**
- Anti-PEG acridan stock **Store ≤ -70°C**
- 20 kDa PEG-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
- Micro-Plate incubator/shaker
- Luminometer capable of simultaneous injection & measurement
- Curve fitting software

STORAGE

Store the HRP conjugate, acridan conjugate and PEG 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 PEGylated molecule that they are studying (see next section). We provide a stock of 20 kDa PEG-amine so that it can be used to demonstrate that the kit works appropriately.

[1] Sensitivity varies with PEG chain molecular weight and the extent of PEGylation.
[2] The SPARCL technology was developed by Lumigen Corp.
[3] The plate provided with the kit has been treated with a reagent that reduces background chemiluminescence. Untreated plates cannot be used.

Life Diagnostics, Inc., P.O. Box 5205, West Chester, PA 19380
610-431-7707 – 610-431-7818 (Fax)
info@lifediagnostics.com – www.lifediagnostics.com

1. Label 8 polypropylene tubes as 10, 2.5, 0.625, 0.156, 0.0391, 0.0098, 0.0024 and 0.0006 ng/ml.
2. Into the tube labeled 10 ng/ml, pipette the volume of diluent detailed on the 20 kDa PEG-amine stock vial label. Then add the indicated volume of stock and mix gently. This provides the 10 ng/ml standard.
3. Dispense 150 µl of diluent into the tubes labeled 2.5, 0.625, 0.156, 0.0391, 0.0098, 0.0024 and 0.0006 ng/ml.
4. Pipette 50 µl of the 10 ng/ml PEG standard into the tube labeled 2.50 ng/ml and mix. This provides the 2.5 ng/ml PEG standard.
5. Similarly prepare the remaining standards by 4-fold serial dilution.

TIPS FOR PREPARATION OF STANDARDS

As discussed in the "principle of the assay" section, the sensitivity of the assay depends on PEG chain length and extent of PEGylation (Figures 1 & 2, Table 1). For these reasons, it is essential that the PEG or PEGylated 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.

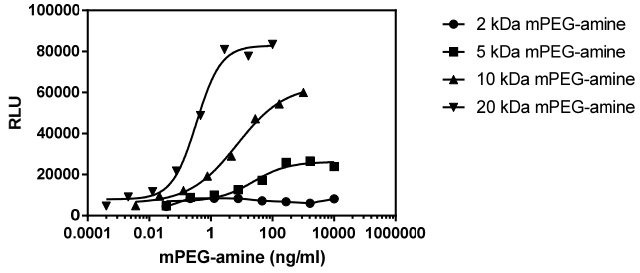


Figure 1. Standard curves obtained with mPEG-amines of varying molecular weights.

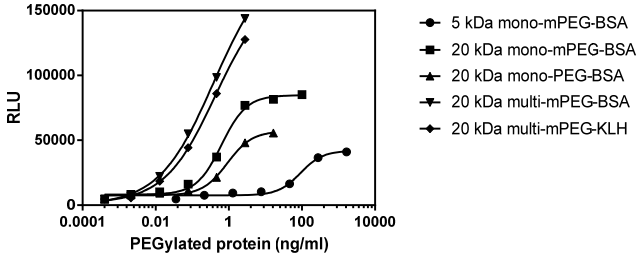


Figure 2. Standard curves obtained with mono- and multi-PEGylated proteins.

PEG	IC_{50} (ng/ml)
2 kDa mPEG-amine	-
5 kDa mPEG-amine	24.0
10 kDa mPEG-amine	7.2
20 kDa mPEG-amine	0.34
5 kDa mono-mPEG-BSA	91.5
20 kDa mono-mPEG-BSA	0.63
20 kDa mono-PEG-BSA	0.91
20 kDa multi-mPEG-BSA	0.38
20 kDa multi-mPEG-KLH	0.42

Table 1. IC_{50} values of PEG-amines and PEGylated 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 20 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 64-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

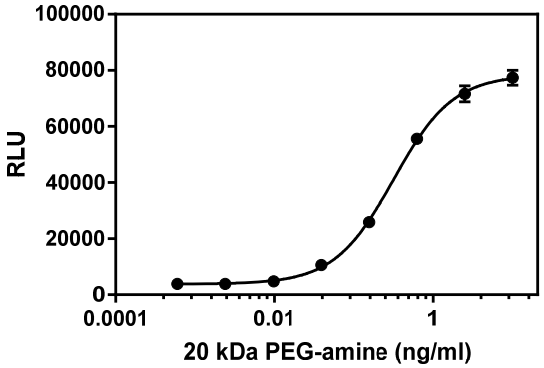
1. Before calculating results, review the raw data. If artefacts (RLU spikes) are apparent immediately after injection of trigger solution, eliminate that portion of the luminescence profile from analysis for all wells. We routinely use the sum of RLU values from a 100-980 ms data collection window.

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 PEG or PEGylated 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 $\log_{10}$ 20 kDa PEG-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 20 kDa PEG-amine should be approximately 0.3 ng/ml.

20 kDa PEG (ng/ml)	RLU
10	77348
2.5	71629
0.625	55590
0.156	25911
0.0391	10617
0.0098	4808
0.0024	3894
0.0006	3871



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