

PEGylated protein ELISA kit

Catalog # ADI-900-213

1x96 well Colorimetric Detection Kit

For use with serum, plasma, tissue extracts and other biological samples



Check our website for additional protocols, technical notes and FAQs.



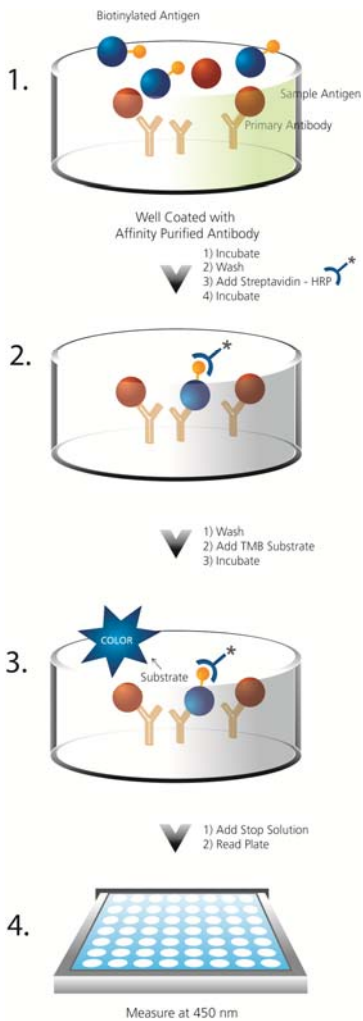
For proper performance, use the insert provided with each individual kit received.

Table of Contents

<u>2</u>	<u>Introduction</u>
<u>3</u>	<u>Principle</u>
<u>4</u>	<u>Materials Supplied</u>
<u>5</u>	<u>Storage</u>
<u>5</u>	<u>Other Materials Needed</u>
<u>6</u>	<u>Reagent Preparation</u>
<u>8</u>	<u>Sample Handling</u>
<u>10</u>	<u>Assay Procedure</u>
<u>11</u>	<u>Calculation of Results</u>
<u>12</u>	<u>Typical Results</u>
<u>13</u>	<u>Performance Characteristics</u>
<u>18</u>	<u>References</u>
<u>20</u>	<u>Warranty</u>

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INTRODUCTION



The PEGylated protein ELISA kit is compatible with plasma, serum, tissue lysate samples and other biological samples. The competitive assay is specific to the backbone of poly(ethylene glycol) (PEG) and has been validated for use with a wide variety of PEG molecules including linear and branched forms as well as free and conjugated forms. As expected, the assay is more sensitive to higher molecular weight PEG molecules due to the repeating characteristics of the backbone. The assay uses a monoclonal antibody to PEG pre-coated on a microtiter plate. The antibody binds in a competitive manner the PEG or PEGylated protein in the sample or the PEG covalently linked to biotin. After incubation at room temperature the excess reagents are washed away and a streptavidin HRP conjugate is added to bind to the biotinylated PEG bound to the antibody. After a second wash to remove excess HRP, substrate is added. An HRP-catalyzed reaction generates a blue color in the solution. The reaction is stopped with stop solution and the resulting yellow color is read at 450nm. The amount of signal is inversely proportional to the concentration of antigen.

Poly(ethylene glycol) (PEG) is a widely used polymer in drug delivery systems and is often directly conjugated to a drug therapeutic. PEGylation of drug therapeutics is desirable as it has been found to increase the retention time, reduce the immunogenicity and increase stability towards metabolic enzymes.¹ The PEGylated Protein ELISA kit is applicable for drug development and pharmaceutical manufacturing applications including drug formulations, pharmacokinetics analysis, drug comparison, lead candidate identification, lot release criteria and in-process QC studies. At this time, there are no long-term studies revealing the fate of this polymer in the body.² The need for toxicology studies that would determine the accumulation of the metabolized PEGylated therapeutics in various tissues has been identified.³

Principle

1. Samples and standards are added to wells coated with a monoclonal antibody specific for PEG. A solution of PEG conjugated to Biotin is then added.
2. During a simultaneous incubation at room temperature the coated antibody binds in a competitive manner, the PEG in the sample or the biotinylated PEG.
3. The plate is washed, leaving only bound PEG on the plate. A solution of HRP conjugate is added to each well. This binds the biotinylated PEG captured on the plate. The plate is again incubated.
4. The plate is washed to remove excess HRP conjugate. TMB substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is inversely proportional to the amount of antigen in the sample.



Do not mix components from different kit lots or use reagents beyond the kit's expiration date.



Activity of conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.



Concentrated Wash Buffer 3 may produce an abundant precipitate if it is frozen, warming in 37°C water bath will facilitate this precipitate to go back into solution and does not have any adverse effects on the assay.



Protect substrate from prolonged exposure to light.



Stop solution is caustic. Keep tightly capped.

MATERIALS SUPPLIED

- 1. PEG Microtiter Plate, One Plate of 96 Wells**
Catalog No. 80-2419
A plate using break-apart strips coated with a monoclonal antibody specific to PEG
- 2. PEG-BSA (Bovine Serum Albumin) Assay Control**
1 x 0.05 mL
Catalog No. 80-2403
One vial containing a solution of 22.5 µg/mL PEGylated BSA
- 3. Biotinylated PEG, 5 mL**
Catalog No. 80-2397
A red solution of Biotinylated PEG
- 4. Assay Buffer 39 Concentrate, 10x , 25 mL**
Catalog No. 80-2420
Tris buffered saline containing proteins and detergents
Assay Buffer 39 is specially formulated for compatibility with the ELISA
- 5. PEG ELISA Conjugate, 500x, 0.05 mL**
Catalog No. 80-2401
A blue solution of streptavidin conjugated to horseradish peroxidase
- 6. Wash Buffer 3 Concentrate, 20x, 2 x 25 mL**
Catalog No. 80-2421
Phosphate buffered saline containing detergents
Wash Buffer 3 is specially formulated for compatibility with the ELISA
- 7. PEG ELISA Conjugate Diluent, 12 mL**
Catalog No. 80-2422
A blue solution of tris buffered saline containing proteins
- 8. TMB Substrate, 10 mL**
Catalog No. 80-0350
A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide
- 9. Stop Solution 2, 10 mL**
Catalog No. 80-0377
A 1N solution of hydrochloric acid in water. Keep tightly capped
- 10. Plate Sealer, 2 each**
Catalog No. 30-0012

STORAGE

All components of this kit are stable at 4°C until the expiration date.

OTHER MATERIALS NEEDED

1. Pure or well characterized PEGylated molecule of interest of known concentration to be used as the standard in the assay (see page 5)
2. Distilled or deionized water
3. Precision pipettes for volumes between 5 μL and 1,000 μL
4. Disposable polypropylene test tubes for dilution of samples and standards.
5. Repeater pipettes for dispensing 50 μL and 100 μL
6. Disposable beakers for diluting buffer concentrates
7. Graduated cylinders
8. Microplate shaker
9. Absorbent paper for blotting
10. Microplate reader capable of reading at 450 nm
11. Software (such as AssayBlaster™ catalog number ADI-28-0002) for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit.



If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

REAGENT PREPARATION

1. Assay Buffer 39, 1x

Prepare the Assay Buffer by diluting 10 mL of the supplied Assay Buffer Concentrate with 90 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. Wash Buffer 3, 1x

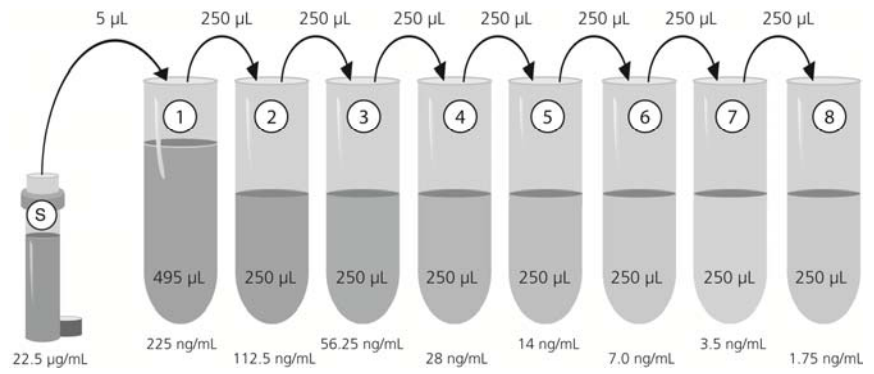
Prepare the wash buffer by diluting 25 mL of the supplied Wash Buffer Concentrate with 475 mL of deionized water. This can be stored at room temperature until the kit expiration or for 3 months, whichever is earlier. Concentrated Wash Buffer 3 may produce an abundant precipitate if it is frozen, warming in 37°C water bath will facilitate this precipitate to go back into solution and does not have any adverse effects on the assay.

3. PEGylated Protein Standard Curve

The end-user will need to determine the dynamic range and sensitivity of the assay to each PEGylated protein of interest. We recommend serially diluting the PEGylated protein standard in 1x assay buffer over a large concentration range covering low $\mu\text{g/mL}$ to low ng/mL concentrations. To obtain relevant sample concentrations the PEGylated standard must be identical to that present in the samples. The PEGylated BSA Assay Control provided in the kit is recommended to be used as a positive control to ensure the assay is running as expected, and not as a standard for concentration determination of samples.

4. PEGylated BSA Assay Control

A PEGylated BSA is provided in the kit and may be used as an assay control. A known amount of BSA has been conjugated with multiple PEG molecules. We suggest running the assay control from 1.75 – 225 ng/mL (see typical results on page 10). Label eight polypropylene test tubes #1 through #8. Pipet 495 μL 1x assay buffer into tube #1 and 250 μL into tubes #2-#8. Carefully add 5.0 μL of the PEGylated BSA Assay Control stock solution to tube #1 and gently vortex. Take 250 μL of the PEGylated BSA solution in tube #1 and add it to tube #2 and gently vortex. Repeat this for tubes #3 through #8. Dilute fresh controls daily.



5. PEG ELISA Conjugate Dilution

Dilute PEG ELISA conjugate 500-fold, 10 µL PEG ELISA Conjugate in 5 mL PEG ELISA Conjugate Diluent. This dilution should be made fresh on each day the assay is run.

SAMPLE HANDLING

The Enzo Life Sciences PEGylated Protein ELISA kit is compatible with a variety of biological samples. Samples containing a visible precipitate must be clarified prior to use in the assay. A variety of tissue homogenization buffers have been tested for assay tolerance (see page 12). Samples diluted sufficiently into the assay buffer can be read directly from a standard curve.

A minimum 1:8 dilution is recommended for serum and plasma. These are the minimum recommended dilutions to remove matrix interference in the assay. Do not use grossly hemolyzed or lipemic specimens.

Plasma Preparation

1. Collect whole blood in vacutainer tube containing EDTA.
2. Centrifuge at 1000 x g for 15 minutes at 4°C.
3. Place supernatant in a clean tube.
4. The supernatant may be divided into aliquots and stored at or below -20°C, or used immediately in the assay.
5. Avoid repeated freeze-thaw cycles.

Serum Preparation

1. Collect whole blood in appropriate tube.
2. Incubate upright at room temperature for 30-45 minutes to allow clotting to occur.
3. Centrifuge at 1000 x g for 15 minutes at room temperature. Do not use brake.
4. Without disturbing the cell layer, place supernatant into a clean tube.
5. The supernatant may be divided into aliquots and stored at or below -20°C, or used immediately in the assay.
6. Avoid repeated freeze-thaw cycles.


Tissue extract Preparation


We have supplied a general protocol for tissue homogenization. The end-user will need to validate the assay for each PEGylated molecule. Refer to tissue homogenization buffer compatibility on page 12. Care should be taken to avoid polyoxyethylene derivatives in homogenization/extraction buffers as these will interfere with the assay. The end-user will need to determine the amount of tissue needed for analysis.


1. Transfer tissue sample to appropriate sized tube for homogenization with extraction buffer.
2. For mechanical homogenizer, disrupt the tissue with three pulses of 3-4 seconds each. For manual dounce homogenizer, complete a minimum of 5 passes of the pestle past the buffer/tissue volume, or until tissue


appears completely homogenized. Keep samples on ice while completing all preparations.


3. Pellet out tissue/cellular debris by centrifugation at 14000g for 10 minutes at 4°C and transfer supernatant to a clean tube.
4. Prepare tissue homogenates for use in the PEGylated protein ELISA by diluting the sample in assay buffer. End-user will need to determine appropriate dilution.

 Bring all reagents to room temperature for at least 30 minutes prior to opening.

 Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

 All standards and samples should be run in duplicate.

 Pipet the standards and samples to the bottom of the wells.

 Pipet the reagents to the sides of the wells to avoid possible contamination.

ASSAY PROCEDURE

Refer to the Assay Layout Sheet to determine the number of wells to be used.

1. Bring all reagents to room temperature for at least 30 minutes prior to opening.
2. Refer to the assay layout sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C .
3. Serially dilute PEGylated protein standards in assay buffer (see page 5).
4. Dilute PEGylated BSA assay control (1:2 serial dilutions) in assay buffer.
5. Allow samples to thaw on ice prior to use. Dilute samples 1:8 in assay buffer.
6. Pipet 50 µL of assay buffer into the Bo (0 ng/mL standard) wells and 100 µL assay buffer into Non-Specific Binding (NSB) wells.
7. Pipet 50 µL of Assay Controls #1 through #8 into the appropriate wells.
8. Pipet 50 µL of Standards into the appropriate wells.
9. Pipet 50 µL of the Samples into the appropriate wells.
10. Pipet 50 µL of Biotinylated PEG into all wells, except NSB and Blank wells.
11. Seal the plate. Incubate at room temperature on a plate shaker for 1 hour at ~500 rpm*.
12. Empty the contents of the wells and wash by adding a full well volume (~300 µL) of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
13. Pipet 100 µL of PEG ELISA Conjugate into all wells, except Blank.
14. Seal the plate. Incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm*.
15. Wash as above.
16. Pipet 100 µL of TMB Substrate solution into each well.
17. Seal the plate. Incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm*.
18. Pipet 100 µL Stop Solution 2 to each well.

19. Read the optical density at 450nm.
 - * The plate shaker speed was based on a BellCo Mini Orbital Shaker (model no. 7744-08096). The actual speed of the plate shaker should be such that the liquid in the plate wells mixes thoroughly, but does not splash out of the well.

CALCULATION OF RESULTS



Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of PEGylated protein in the samples. We recommend that the data be handled by an immunoassay software package utilizing a four parameter logistic curve fitting program (such as AssayBlaster™, catalog number ADI-28-0002). Such software is often supplied by plate reader manufacturers.

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

$$\text{Average Net OD} = \text{Average OD} - \text{Average NSB OD}$$

2. Calculate the binding of each pair of standard as well as a percentage of the maximum binding wells (Bo), using the following formula:

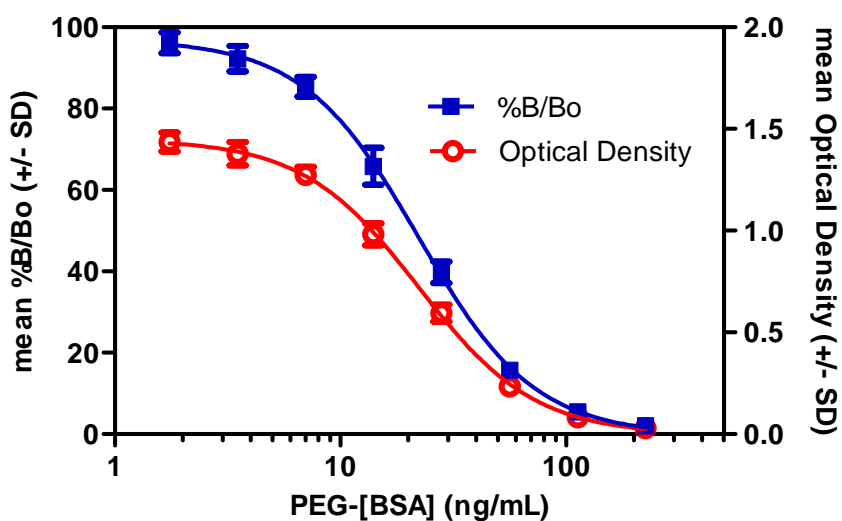
$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

Samples with concentrations outside of the standard curve range will need to be reanalyzed using a different dilution.

TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate the results from another assay.

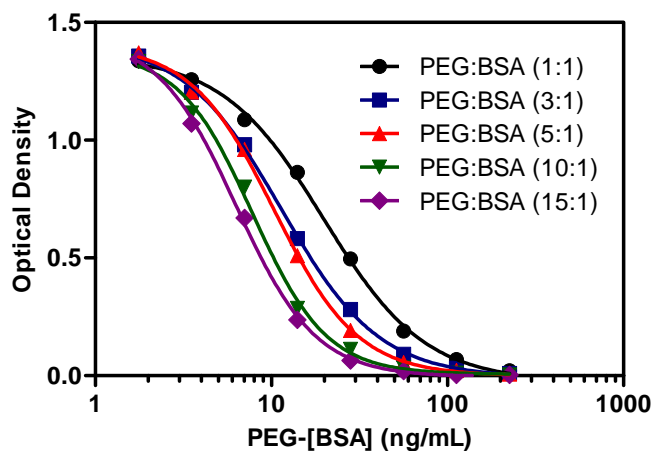
Sample	Mean OD	Net OD	%B/Bo	PEGylated BSA (ng/mL)
NSB	0.044	---	---	---
Bo	1.539	1.495	100	0
S1	0.074	0.030	2.01	225
S2	0.126	0.082	5.48	113
S3	0.279	0.235	15.7	56.3
S4	0.638	0.594	39.7	28.1
S5	1.026	0.982	65.7	14.1
S6	1.319	1.275	85.3	7.03
S7	1.422	1.378	92.2	3.52
S8	1.480	1.436	96.1	1.76



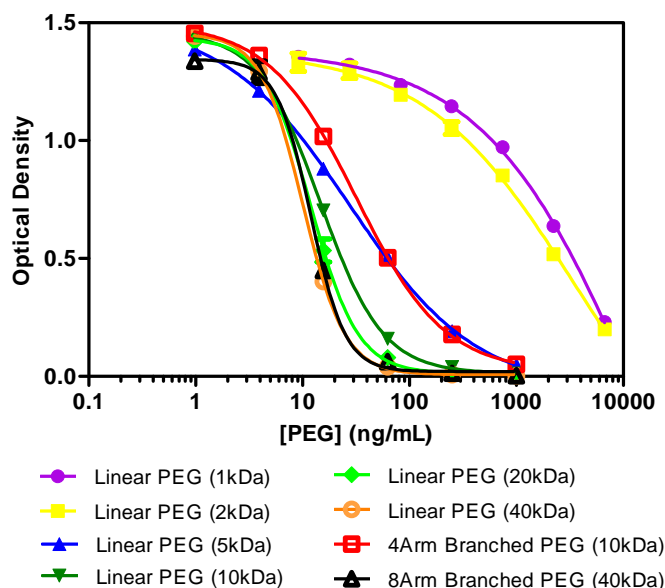
PERFORMANCE CHARACTERISTICS

Sensitivity & Specificity

The sensitivity of the assay is dependent upon the molecular weight of PEG and the quantity of PEG molecules conjugated to a target molecule. An experiment was carried out by preparing multiple conjugates using 10 kDa linear PEG offered at 1, 3, 5, 10 and 15-fold molar excess to BSA. The PEG-BSA conjugates were diluted in assay buffer and run in the assay. The OD at 450 nm was plotted as a function of BSA concentration. The assay shows increased sensitivity with increased offerings of PEG to BSA.



The detector antibody is specific to the backbone of PEG and therefore, is able to bind PEG molecules of greater molecular weight with increased sensitivity. Several PEG molecules of different molecular weight and form have been titrated in the assay. The OD at 450 nm was plotted as a function of PEG concentration. The ED_{50} of each dose response curve was tabulated below. The assay has been shown to detect linear PEG between the size of 1 and 40kDa and 4-arm and 8-arm branched PEG of 10 and 40kDa molecular weight, respectively.



Free PEG	ED50 (ng/mL)
mPEG 1kDa	1711
mPEG 2kDa	1146
mPEG 5kDa	30
mPEG 10kDa	16
mPEG 20kDa	12
mPEG 40kDa	10
4Arm-Branched PEG 10kDa	37
8Arm-Branched PEG 40 kDa	11

Interference

Polyoxyethylene derivatives should not be used in this assay due to structural similarity to PEG. These derivatives include, but are not limited to: Tween, Triton, NP-40, Pluronic F-127 and Genapol X-080.

Several tissue homogenization buffers and detergents were tested in the assay for compatibility. PEGylated BSA was spiked into serial dilutions of Tissue Protein Extraction Reagent (T-PER) (Thermo, Cat. #78510), Mammalian Protein Extraction Reagent (M-PER) (Thermo, Cat. #78501), RIPA cell lysis buffer (CLB) and sodium dodecyl sulfate (SDS) and compared to assay buffer spiked to the same level. Commercially available buffers, T-PER and M-PER did not interfere in the assay; whereas, RIPA cell lysis buffer should not be used due to the high level of interference from buffer components*. SDS is compatible in the assay at or below. 0.125%.

Dilution (1:x)	T-PER	M-PER	RIPA CLB*	Percent	SDS
2	92	102	5.3	1.0	6.5
4	98	83	5.1	0.5	11
8	99	96	5.4	0.25	83
16	100	96	5.8	0.13	96
32	101	99	7.1	0.06	103
64	100	99	11	0.03	99

**50mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1.0% Triton X-100, 0.1% SDS, 1% sodium deoxycholate*

Linearity

Pools of five unique plasma and serum samples of human, mouse and rat origin were prepared. PEGylated BSA was spiked into prepared samples and serially diluted in assay buffer. Sample dilutions were analyzed in the assay. Concentrations were assigned off a PEGylated BSA standard curve prepared in assay buffer. Dilutional linearity was calculated by dividing the assigned concentration at dilution over the assigned concentration at the 1:16 dilution, and multiplying by 100.

Dilution (1:x)	Human Plasma	Human Serum	Mouse Plasma	Mouse Serum	Rat Plasma	Rat Serum
2	102%	96%	91%	99%	116%	116%
4	103%	103%	106%	110%	120%	122%
8	102%	106%	106%	109%	117%	123%
16	100%	100%	100%	100%	100%	100%

Parallelism

To achieve parallel dose response curves of sample analyte to assay standard, end-user must match the sample analyte to a PEGylated protein standard, i.e. same PEG molecule used in-vitro or in-vivo studies matches PEGylated protein standard (both molecular weight and form). The end-user may use the provided PEG-BSA assay control for semi-quantitative measurements.

Spike & Recovery

PEGylated IgG was spiked separately into 1:4 and 1:8 diluted plasma and serum (human, mouse and rat) at 1 and 5ng/mL. Each spiked sample was run in the assay against a PEGylated IgG standard curve that was prepared in a matching sample matrix diluted 1:8 in assay buffer. The percent recovery was determined by dividing the resulting value of each spiked sample matrix by the concentration of the corresponding theoretical spiked concentration, and multiplying by 100.

Species: Rat	Dilution:	1:4	1:8
	Spike Conc.	Mean	Mean
Sample	(ng/mL)	%Recovery	%Recovery
Plasma	1.0	110	95
	5.0	98	89
Serum	1.0	168	139
	5.0	110	103

Species: Mouse	Dilution:	1:4	1:8
	Spike Conc.	Mean	Mean
Sample	(ng/mL)	%Recovery	%Recovery
Plasma	1.0	121	101
	5.0	144	85
Serum	1.0	168	115
	5.0	123	106

Species: Human	Dilution:	1:4	1:8
	Spike Conc.	Mean	Mean
Sample	(ng/mL)	%Recovery	%Recovery
Plasma	1.0	122	90
	5.0	179	88
Serum	1.0	115	77
	5.0	92	77

Precision

Intra-assay precision was determined by assaying 16 replicates of two buffer controls containing PEG-BSA in a single assay.

Sample	PEGylated BSA Conc. (ng/mL)	%CV
1	25	3.4
2	50	4.2

Inter-assay precision was determined by back-calculating three standards off 16 standard curves produced in multiple assays over several days.

Expected PEG- BSA Conc. (ng/mL)	Back-calculated PEG-BSA Conc. (ng/mL)	%Error	%CV
112.5	113	0.4	2.4
28.1	27.7	1.4	1.3
7.03	7.25	3.1	5.7

REFERENCES

1. Harris, J.M. et al. (2001) Clin Pharmacokinet. 40, 539-551
2. Schubert. U. S. et al. (2010) Angew. Chem. Ed. 49, 6288-6380
3. Veronese, F.M. and Pasut, G. (2005) DDT. 10, 1451-1458

NOTES



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