

## An ELISA Kit for the measurement of PEGylation and quantification of PEG and PEGylated molecules

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### INTRODUCTION

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 increases the retention time, reduces the immunogenicity and improves resistance to proteases.<sup>1</sup> We report the development of a sensitive immunoassay for the quantitative detection of PEG and PEGylated molecules using a colorimetric 96-well microtiter plate format. 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. PEG is not biodegradable and there are no long-term studies revealing the fate of this polymer in the body.<sup>2</sup> The need for toxicology studies that would determine the accumulation of the metabolized PEGylated therapeutics in various tissues has been identified.<sup>3</sup> The PEGylated Protein ELISA may enable such toxicology studies.

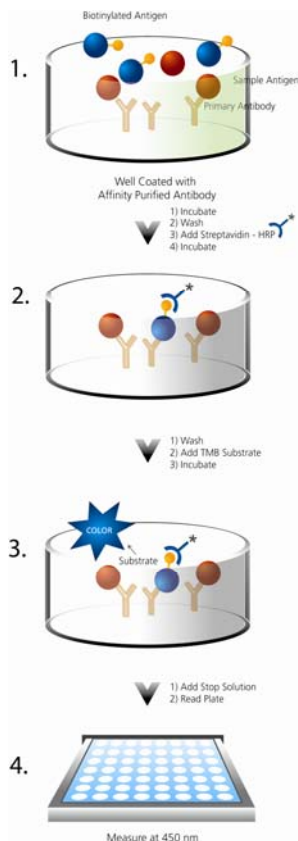
In this format, a monoclonal antibody, specific to the linear backbone of PEG, was coated through passive absorption on high binding microtiter plate. During a simultaneous incubation, the antibody binds in a competitive manner to biotinylated- PEG and the PEGylated molecule in the sample. After washing, a solution of peroxidase (HRP) conjugated streptavidin is added to each well, binding to the captured biotinylated- PEG. A substrate solution is added and the resulting HRP-catalyzed reaction is read at 450 nm. The amount of signal is indirectly proportional to the concentration of PEG in the sample. The assay is designed to measure free PEG of different molecular weights and forms and PEGylated molecules in a wide range of sample matrices. PEGylated-BSA was measured from 1.76 – 225 ng/mL. The intra-assay precision ranged from 3.4 to 4.2% and the inter-assay precision ranged from 1.3 to 5.7% CV. The assay reactivity to conjugated PEGylated-BSA increased with molar increases of PEG derivative offered to BSA, suggesting the assay may be useful in determining conjugation efficiency. Dilutional linearity was achieved at the 1:2 dilution in assay buffer for serum and plasma from human, mouse and rat origin. PEGylated-IgG was spiked separately into plasma and serum (human, mouse and rat origin) at 1 and 5 ng/mL and compared to PEGylated-IgG standard curve prepared in a matching sample matrix. The mean recovery of 1 and 5 ng/mL spike in 1:8 diluted plasma was 90 and 88% (human), 101 and 85% (mouse) and 95 and 89% (rat), respectively. The mean recovery of the 1 and 5 ng/mL spike in 1:8 diluted serum was 77 and 77% (human), 115 and 106% (mouse) and 139 and 103% (rat), respectively. Several tissue homogenization buffers and detergents were tested in the assay for compatibility. Mammalian Protein Extraction Reagent (Thermo, Cat. #78501) and Tissue Protein Extraction Reagent (Thermo, Cat. #78510) were found to not interfere in the assay. Sodium dodecyl sulfate (SDS) was also compatible in the assay at or below 0.125%.

PRODUCT	PRODUCT #	SIZE
PEGylated Protein ELISA Kit	ADI-900-213	1 x 96 wells

## PROCEDURES

### Assay Protocol

- (1) Standard or samples and biotinylated PEG are added to wells pre-coated with mouse anti-PEG IgG antibody and incubated at room temperature. During this primary incubation, the antibody binds, in a competitive manner, the PEG in the sample or the biotinylated PEG.
- (2) The plate is washed, leaving only bound PEG. A solution of streptavidin conjugated to horseradish peroxidase is added to each well, to bind to the biotinylated PEG.
- (3) The plate is washed to remove excess HRP conjugate. TMB substrate solution is added to each well. An HRP-catalyzed reaction generates a blue color in the solution.
- (4) 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 PEG in the sample.

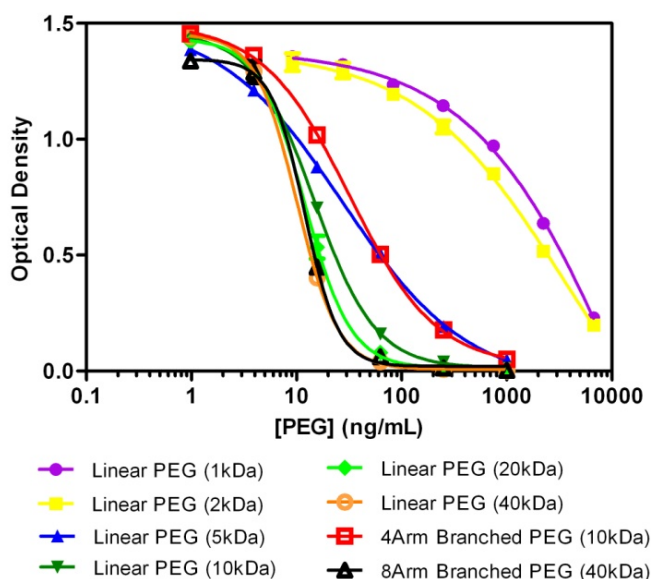


## RESULTS

### Sensitivity and Specificity

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 (Figure 1).

**Figure 1.** Assay specificity to different molecular weights and forms of PEG



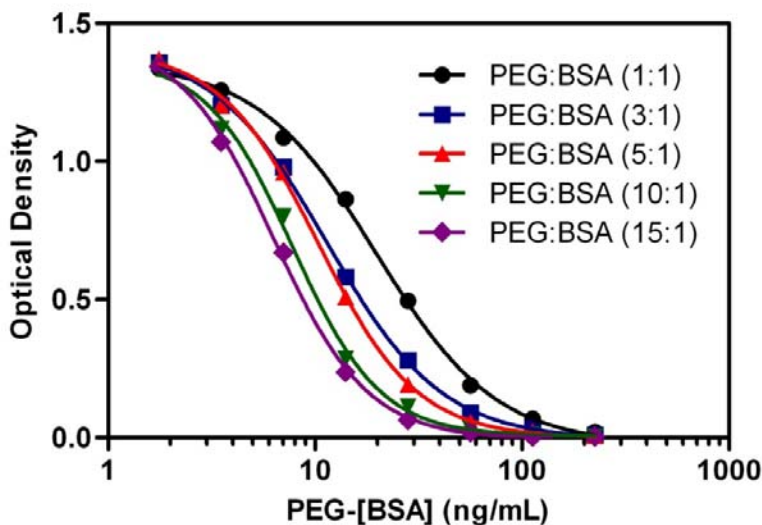
The ED50 of each dose response curve was tabulated (Table 1).

**Table 1.** Calculated ED50 of free PEG dose response curves

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 40kDa	11

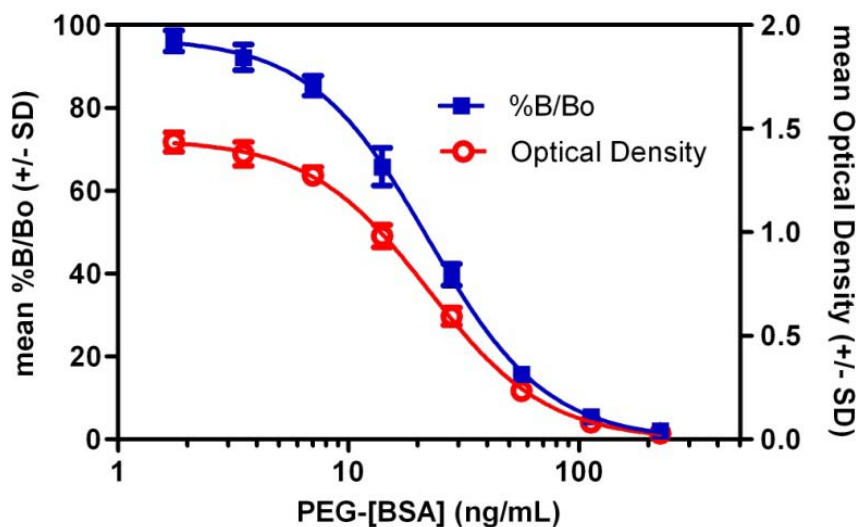
The sensitivity of the assay is dependent upon the molecular weight of PEG and the quantity of PEG molecules conjugated to a target molecule. An experiment was carried out by preparing multiple conjugates using 10 kDa linear PEG offered at 1, 3, 5, 10 and 15-fold molar excess to BSA. Unconjugated PEG was removed by centrifugal filtration. The PEG-BSA conjugates were normalized to BSA concentration, serially diluted in assay buffer and analyzed in the assay. The OD at 450 nm was plotted as a function of BSA concentration (Figure 2).

**Figure 2.** Different molar offerings of PEG to BSA conjugates



10 kDa PEG was conjugated to BSA. Concentration of the resulting conjugate was based on A280 reading using the extinction coefficient of BSA. The conjugate was serially diluted in assay buffer from 1.76 – 225 ng/mL and run in the assay. The OD at 450 nm and percent binding were plotted as a function of BSA concentration (Figure 3).

**Figure 3.** PEGylated-BSA standard curve



## Tissue homogenization buffer interference

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. Concentrations of spiked samples and control was determined from the PEGylated-BSA standard curve. Percent recovery was calculated by dividing the assigned concentration in the sample by the concentration assigned in the spiked assay buffer control, and multiplying by 100 (Table 2).

**Table 2.** Assay tolerance to tissue homogenization buffers and detergents

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

## Plasma and serum dilutional 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 (Table 3).

**Table 3.** Plasma and serum dilutional linearity

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%

## Spike & Recovery

PEGylated-IgG was spiked separately into 1:8 diluted plasma and serum (human, mouse and rat origin) at 1 and 5 ng/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 (Table 4).

**Table 4.** Spike & recovery of PEGylated-IgG in plasma and serum

Species:	Human	Dilution:	1:8
Sample	Spike Conc. (ng/mL)		Mean %Recovery
Plasma	1.0		90
	5.0		88
Serum	1.0		77
	5.0		77
Species:	Mouse	Dilution:	1:8
Sample	Spike Conc. (ng/mL)		Mean %Recovery
Plasma	1.0		101
	5.0		85
Serum	1.0		115
	5.0		106
Species:	Rat	Dilution:	1:8
Sample	Spike Conc. (ng/mL)		Mean %Recovery
Plasma	1.0		95
	5.0		89
Serum	1.0		139
	5.0		103

### Assay Reproducibility

Inter-assay precision was determined by back-calculating three standards off 16 standard curves produced in multiple assays over several days (Table 5). Intra-assay precision was determined by assaying 16 replicates of two buffer controls containing PEG-BSA in a single assay (Table 6).

**Table 5.** Inter-assay precision

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

**Table 6.** Intra-assay precision

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

## CONCLUSIONS

1. The ELISA detected various PEG molecules of both linear and branched forms with greater sensitivity towards higher molecular weight molecules.
2. The assay shows increased sensitivity with increased molar offerings of PEG to BSA conjugates. PEGylated-BSA was detected from 1.76 – 225 ng/mL.
3. The assay is compatible with commercially available, Mammalian and Tissue Protein Extraction Reagent buffers (Thermo Fisher) and sodium dodecyl sulfate at or below 0.125%.
4. Lysis buffers containing Triton-100 should not be used due to the high level of interference.
5. Dilutional linearity of spiked plasma and serum samples of human, mouse and rat origin was achieved at a 1:2 dilution in assay buffer.
6. The recovery of 1 and 5 ng/mL PEGylated-IgG spiked in 1:8 diluted plasma was 90 and 88% (human), 101 and 85% (mouse) and 95 and 89% (rat), respectively.
7. The recovery of 1 and 5 ng/mL PEGylated-IgG spiked in 1:8 diluted serum was 77 and 77% (human), 115 and 106% (mouse) and 139 and 103% (rat), respectively.
8. Inter-assay precision was determined to be 3.4 – 4.2 %CV.
9. Intra-assay precision was determined to be 1.3 – 5.7 %CV.

## REFERENCES

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