Version 14 Last updated 27 September 2019

ab133065 – PEGylated Protein ELISA Kit

For quantitative detection of PEGylated protein in serum, plasma, tissue extracts and other biological samples.

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

Table of Contents

1. Overview

Abcam's PEGylated protein in vitro competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of PEGylated protein in plasma, serum, tissue lysate samples and other biological samples.

A monoclonal antibody specific to PEGylated protein has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with a solution of PEGylated protein conjugated to biotin. The plate is washed to remove unbound reagents. A solution of streptavidin-HRP conjugate is then added. After further incubation the excess reagents are washed away and TMB substrate is added, which is catalyzed by HRP to generate a blue color. A stop solution changes this color from blue to yellow, and the intensity of this yellow coloration is inversely proportional to the amount of PEGylated protein captured in the plate.

The competitive assay is specific to the backbone of poly(ethylene glycol) (PEGylated protein) and has been validated for use with a wide variety of PEGylated protein molecules including linear and branched forms as well as free and conjugated forms. As expected, the assay is more sensitive to higher molecular weight PEGylated protein molecules due to the repeating characteristics of the backbone. The assay uses a monoclonal antibody to PEGylated protein pre-coated on a microtiter plate. The antibody binds in a competitive manner the PEGylated protein or PEGylated protein in the sample or the PEGylated protein 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 PEGylated protein bound to the antibody. After a second wash to remove excess HRP, substrate is added. A 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) (PEGylated protein) 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. 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. The need for toxicology studies that would determine the accumulation of the metabolized PEGylated therapeutics in various tissues has been identified.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed.

Add samples and Biotinylated antigen to appropriate wells. Incubate at room temperature.

Wash and add prepared labeled Streptavidin HRP-conjugate to appropriate wells. Incubate at room temperature.

Wash and add TMB substrate to each well. Incubate at room temperature.

Add Stop Solution to each well. Read immediately.

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide: www.abcam.com/assaykitguidelines
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors.

6. Materials Supplied

Item	Quantity	Storage condition
PEGylated Protein Microplate (12 x 8 wells)	96 wells	+4°C
Biotinylated PEGylated Protein	5 mL	+4°C
PEGylated BSA Control	50 µL	+4°C
10X Assay Buffer 39	25 mL	+4°C
20X Wash Buffer 3 Concentrate	2 x 25 mL	+4°C
TMB Substrate	10 mL	+4°C
Stop Solution 2	10 mL	+4°C
PEGylated Protein Conjugate Diluent	12 mL	+4°C
500X PEGylated Protein Conjugate	50 µL	+4°C
Plate Sealer	2 units	+4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Pure or well characterized PEGylated molecule of interest of known concentration to be used as the standard in the assay
- Distilled or deionized water
- Precision pipettes for volumes between 5 µL and 1,000 µL
- Disposable polypropylene test tubes for dilution of samples and standards.
- Repeater pipettes for dispensing 50 µL and 100 µL
- Disposable beakers for diluting buffer concentrates
- Graduated cylinders
- Microplate shaker
- Absorbent paper for blotting
- Microplate reader capable of reading at 450 nm

8. Technical Hints

- Standards can be made up in either glass or plastic tubes
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette standards and samples to the bottom of the wells
- Add the reagents to the side of the well to avoid contamination
- This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided
- Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands
- Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. Before using the kit, reagents should be mixed gently (not vortexed) by swirling.
- Prepare only as much reagent as is needed on the day of the experiment.

9.1 PEGylated protein ELISA Conjugate Diluent:

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

9.2 1X Assay Buffer 39:

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.

9.3 1X Wash Buffer 3:

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.

9.4 PEGylated BSA Control:

A PEGylated BSA is provided in the kit and may be used as a positive assay control. A known amount of BSA has been conjugated with multiple PEG molecules. This component is ready to use.

10. Standard Preparation

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 µg/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.

We suggest to assay linear PEG between the size of 1 and 40kDa and 4-arm and 8-arm branched PEG of 10 and 40kDa molecular weight with this kit, as these have been shown to demonstrate specificity and linearity (see Section 17).

11. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples for the most reproducible assay.

- The PEGylated protein 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. 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

11.1 Plasma samples:

- Collect whole blood in vacutainer tube containing EDTA.
- Centrifuge at 1,000 x *g* for 15 minutes at 4°C.
- Place supernatant in a clean tube.
- The supernatant may be divided into aliquots and stored at or below -20°C, or used immediately in the assay.
- Avoid repeated freeze-thaw cycles.

11.2 Serum samples:

- Collect whole blood in appropriate tube.
- Incubate upright at room temperature for 30-45 minutes to allow clotting to occur.
- Centrifuge at 1,000 x *g* for 15 minutes at room temperature. Do not use brake.
- Without disturbing the cell layer, place supernatant into a clean tube.

- The supernatant may be divided into aliquots and stored at or below -20°C, or used immediately in the assay.
- Avoid repeated freeze-thaw cycles.

11.3 Tissue extract samples:

We have supplied a general protocol for tissue homogenization. The end-user will need to validate the assay for each PEGylated molecule. 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.

- Transfer tissue sample to appropriately sized tube for homogenization with extraction buffer.
- 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.
- Pellet out tissue/cellular debris by centrifugation at 14,000 x *g* for 10 minutes at 4°C and transfer supernatant to a clean tube.
- 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.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at 4°C
- For each assay performed, a minimum of 2 wells must be used as blanks, omitting primary antibody from well additions
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay.

	1	2	3	4
A	Std 1	Std 5	B_s	Sample 2
B	Std 1	Std 5	B_s	Sample 2
C	Std 2	Std 6	NSB	Sample 3
D	Std 2	Std 6	NSB	Sample 3
E	Std 3	Std 7	B_0	Sample 4
F	Std 3	Std 7	B_0	Sample 4
G	Std 4	Std 8	Sample 1	Sample 5
H	Std 4	Std 8	Sample 1	Sample 5

Plate layout shows controls, blanks and standards required for each assay. Use additional strips of wells to assay all your samples.

Key:

Bs = Blank; contains TMB substrate.

NSB = Non-specific binding; contains assay buffer, PEGylated conjugate and TMB substrate.

B_0 = 0 pg/mL standard; contains standard diluent, biotinylated PEG, PEGylated conjugate and TMB substrate.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use
- It is recommended to assay all standards, controls and samples in duplicate
- Refer to the recommended plate layout in Section 12 before proceeding with the assay
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 Add 50 µL of Assay Buffer to the B_O wells and 100 µL of Assay Buffer to NSB wells.

13.3 Add 50 µL of prepared standards and diluted samples to appropriate wells.

13.4 Add 50 µL of Biotinylated PEGylated protein to all wells, except NSB and Blank wells.

13.5 Seal the plate. Incubate the plate at room temperature on a plate shaker for 30 minutes at ~500rpm.

13.6 Empty the contents of the wells and wash by adding 300 µL of 1X Wash Buffer 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.

13.7 Add 100 µL of PEGylated Conjugate into each well, except Blank.

13.8 Seal the plate. Incubate the plate at room temperature on a plate shaker for 30 minutes ~500rpm.

13.9 Wash as described in step 13.6.

13.10 Add 100 µL of the TMB Substrate solution to every well.

13.11 Seal the plate and incubate at room temperature for 30 minutes on a plate shaker at ~500rpm.

13.12 Add 100 µL Stop Solution 2 into each well. The plate should be read immediately.

13.13 Read the O.D. absorbance at 450 nm, preferably with correction between 570 and 590 nm.

14. Calculations

14.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

Average Net OD = Average Bound OD - Average NSB OD

14.2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula

$$\text{Percent Bound} = \frac{\text{Average Net OD}}{\text{Average Net } B_0 \text{ OD}} \times 100$$

14.3 Plot the Percent Bound (B/B_0) and the net OD versus concentration of PEGylated protein for the standards. The concentration of PEGylated Protein in the unknowns can be determined by interpolation of net OD values.

A four parameter algorithm (4PL) provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4-parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

15. Typical Data

Typical standard curve - data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.

Sample	Mean OD ($-B_s$)	% Bound	PEGylated BSA ng/mL
NSB	0.044	-	-
Standard 1	0.074	2.01	225
Standard 2	0.126	5.48	112
Standard 3	0.279	15.7	56.3
Standard 4	0.638	39.7	28.1
Standard 5	1.026	65.7	14.1
Standard 6	1.319	85.3	7.03
Standard 7	1.422	92.2	3.52
Standard 8	1.480	96.1	1.76
B_0	1.539	100	0

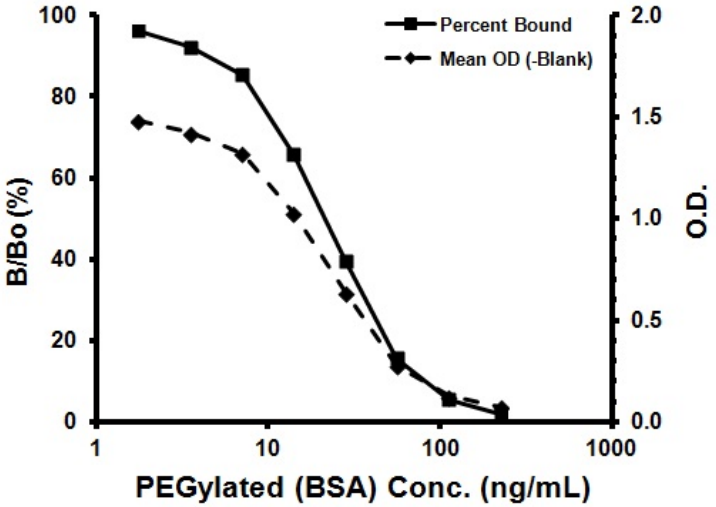

Figure 1. Example PEGylated BSA Standard Curve.

16. Typical Sample Values

SENSITIVITY

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.

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), Mammalian Protein Extraction Reagent (M-PER), 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%.

LINEARITY OF DILUTION

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. The results are shown in the table below.

	Average % Of Expected					
Dilution	Human Plasma	Human Serum	Mouse Plasma	Mouse Serum	Rat Plasma	Rat Serum
1:2	102	96	91	99	116	116
1:4	103	103	106	110	120	122
1:8	102	106	106	109	117	123
1:16	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 PEGylated BSA Control for semi-quantitative measurements.

SAMPLE RECOVERY

PEGylated IgG was spiked separately into 1:4 and 1:8 diluted plasma and serum (human, mouse and rat) 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.

Sample	Dilution: Spike Conc. (ng/mL)	1:4 Mean % Recovery	1:8 Mean % Recovery
Rat Plasma	1	110	95
	5	98	89
Rat Serum	1	168	139
	5	110	103
Mouse Plasma	1	121	101
	5	144	85
Mouse Serum	1	168	115
	5	123	106
Human Plasma	1	122	90
	5	179	88
Human Serum	1	115	77
	5	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 (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

17. Assay Specificity

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 titered in the assay. The OD at 450 nm was plotted as a function of PEG concentration. The ED50 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	1,711
mPEG 2kDa	1,146
mPEG 5kDa	30
mPEG 10kDa	16
mPEG 20kDa	12
mPEG 40kDa	10
4Arm-Branched PEG 10 kDa	37
8Arm-Branched PEG 40 kDa	11

18. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed

19. Notes

Technical Support

Austria
wissenschaftlicherdienst@abcam.com | 019-288-259
France
supportscientifique@abcam.com | 01.46.94.62.96
Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154
Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com
Deutsch: 043-501-64-24 | Français: 061-500-05-30
UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
ca.technical@abcam.com | 877-749-8807
US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823
China
cn.technical@abcam.com | 400 921 0189 / +86 21 2070 0500
Japan
technical@abcam.co.jp | +81-(0)3-6231-0940
Singapore
sg.technical@abcam.com | 800 188-5244

Australia
au.technical@abcam.com | +61-(0)3-8652-1450
New Zealand
nz.technical@abc.com | +64-(0)9-909-7829