PEGylated Molecule Assay Kit **Cat. No. L00458**
Technical Manual No. TM0644 **Updated: 02102015**

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I. DESCRIPTION

PEG (Polyethylene glycol) is a polyether compound with many applications from industrial manufacturing to medicine. PEGylation is a technology that covalently couples non-toxic, hydrophilic PEG to a drug. It is an FDA-approved method for the delivery of protein drugs. PEG modification can reduce the drug immunogenicity and antigenicity. The PEGylated drug decelerates renal excretion, improves stability towards proteolysis and increases its half life in blood. Accurate and sensitive quantification of PEGylated molecules is important for PEG conjugated product development and pharmaceutical study. PEG Antibody is a useful tool for the detection of PEGylated molecules.

GenScript PEGylated Molecule Assay Kit, a 1.5 hour competitive ELISA Kit, is developed for rapid detection of PEGylated molecules. This kit is based on competitive ELISA method. PEG Molecule Plate, which comprises of 8 wells x 12 strips, is demountable. It is a 96-well microtiter plate coated with PEGylated molecule. When Biotin-PEG mAb and PEGylated molecule are added to the well, the PEGylated molecule coated on the plate compete with the PEGylated molecule in the solution to interact with Biotin-PEG mAb. PEG standard control provided in the kit can be used for semiquantitative measurement of PEGylated molecules. To quantitate a PEGylated molecule of interest, operator should use individual PEGylated

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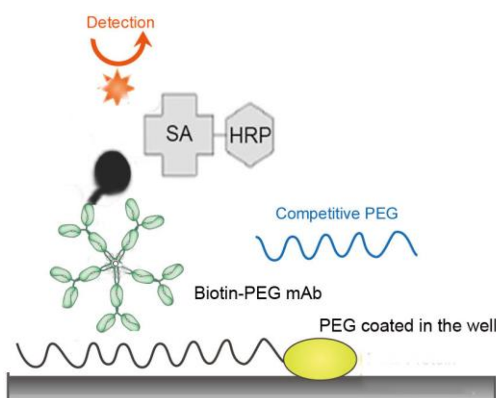
molecule of interest to establish standard curve. (Principle is showed as below.)



Streptavidin-HRP is used for enzyme reaction to develop signal. In the test, PEGylated molecule, Biotin-PEG mAb and Streptavidin-HRP form a complex in the wells on the plate. Other unbound molecules can be removed by wash solution. The Streptavidin-HRP reacts with TMB substrate to develop blue product in the solution. The reaction is stopped by adding stop solution and the color turns yellow which can be read at 450 nm by Microtiter plate reader. Each absorbance value is correlated to each PEGylated molecule concentration in solution. The signal is inversely proportional to the PEGylated molecule concentration in the sample. PEGylated molecule standards of known concentration and the corresponding absorbance values are used to construct a standard curve. With the standard curve, PEGylated molecule amount present in the unknown sample can be calculated by transforming its absorbance value.

II. KIT CONTENT

The kit provides all reagents and solutions for PEGylated molecule detection.

Component	Quantity	Part. No
PEG Molecule Plate	1 plate (8 wells x 12 strips)	458-80
Assay Diluent	60 ml	458-60
Biotin-PEG mAb Stock	100 µl	458-20
Antibody Dilution Buffer	10 ml	458-90
Streptavidin-HRP	12 ml	458-30
PEG Standard Control (10 µg/ml)	50 µl	458-10
20 × Wash Solution	40 ml	458-70
TMB Substrate	12 ml	458-40
Stop Solution	6 ml	458-50
Plate Sealer	2	N/A
User Manual	1	N/A

III. STORAGE

The unopened kit is stable for at least 12 months if stored at 2-8 °C and the opened kit is stable for up to 2 weeks at 2-8 °C. Do not freeze the kit.

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IV. REAGENTS/EQUIPMENTS REQUIRED BUT NOT SUPPLIED

Well characterized PEGylated molecule of interest of known concentration to prepare the standard
Microtiter plate reader capable of measuring absorbance at 450 nm
Automated microplate washer to wash the plate
Deionized or distilled water to dilute 20 x Wash Solution
Graduated cylinder to prepare Wash Solution
Plastic container to store Wash Solution
Tubes to aliquot and dilute samples
Precision pipettes to deliver 10 μl, 100 μl, 200 μl and 1000 μl content
10 μl, 100 μl, 200 μl and 1000 μl pipette tips
Multichannel pipettor
Disposable reagent reservoir
Paper towel
Laboratory timer
Refrigerator to store samples and kit components

V.PROTOCOL

- All reagents in the kit and test samples should be equilibrated to room temperature before test.
- Preliminary experiment should be performed to optimize the sample dilution.

1. Sample Preparation

- Handle serum and plasma samples in accordance with NCCLS (National Committee for Clinical Laboratory Standards) guidelines for preventing transmission of blood-borne infection.
- Assay Diluent is used for sample dilution.
- For serum and plasma sample containing high level of PEGylated molecule, a minimum of 1:100 dilution without heat treatment is recommended to remove matrix interference in the assay.
- For serum and plasma sample containing low level of PEGylated molecule, heat treatment is recommened. A minimum of 1:20 dilution is needed and the diluted samples should be boiled for 5 minutes to remove matrix interference in the assay. Don't boil the sample for a long time to avoid sample getting destroyed.
- The results showed samples with heat treatment have better recovery than those with 1:100 dilution without heat treatment.

Serum: Use a blood separator tube and allow the sample to clot for 30 minutes. Centrifuge for 10 minutes at 1000 x g. Run the assay immediately, otherwise aliquot and store the sample below -20 °C. Avoid repeated thaw-freeze cycle.

Plasma: Treat the blood with citrate, EDTA or heparin as an anticoagulant. Centrifuge for 10 minutes at 1000 x g within 30 minutes for plasma collection. Run the assay immediately. Otherwise aliquot and store the sample below -20 °C. Avoid repeated thaw-freeze cycle.

Cell culture supernatant: Centrifuge the sample to remove the particulate materials. Run the assay immediately, otherwise aliquot and store sample below -20 °C. Avoid repeat thaw-freeze cycle.

2. Reagent Preparation

- If any precipitate is found in the 20 × Wash Solution, incubate the bottle in water bath (up to 50 °C) with occasional mixing until all the precipitate is dissolved.

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1 x Wash Solution: Dilute *20 × Wash Solution* by 1:19 v/v with deionized or distilled water. For example, dilute 40 ml of *20 × Wash Solution* with 760 ml of deionized or distilled water to make 800 ml of 1 × *Wash Solution*. Store at 2-8 °C.

Biotin-PEG mAb Solution: Dilute Biotin-PEG mAb Stock by 1:100 (v/v) with Antibody Dilution Buffer.

PEG Molecule Standards Preparation

To detect PEGylated molecule amount precisely, operator should establish a standard curve with the PEGylated molecule of interest of known concentration. The standard curve established by PEGylated molecule can be used to quantitate the same PEG molecule and is semiquantitative for other PEGylated molecules. Peginterferon Alfa 2A standard curve was established with Peginterferon Alfa 2A as standards in pharmacokinetic study section. It can serve as a typical example for individual PEGylated protein quantitation. Peginterferon Alfa 2A was the protein drug pegylated with a branched 40 kDa PEG chain, which is produced by Roche pharmaceutical company.

- Allow PEG Standard Control to sit for a minimum of 15 minutes at room temparature with gentle agitation prior to making dilution.

2.1 Label seven 1.5 ml Eppendorf tubes with ‘81 ng/ml’, ‘27 ng/ml’, ‘9 ng/ml’, ‘3 ng/ml’, ‘1 ng/ml’, ‘0.333 ng/ml’ and ‘0 ng/ml’.

2.2 Pipette 8.1 μl of *PEG Standard Control* and 991.9 μl of *Sample Diluent* into the tube labeled with ’81 ng/ml’ and vortex it.

2.3 Pipette 400 μl of *Sample Diluent* into the rest empty tubes.

2.4 Pipette 200 μl of 81 ng/ml of PEG molecule solution to the tube labeled with ‘27 ng/ml’ and vortex it to make the standard point of 27 ng/ml.

2.5 Similarly, prepare the rest standard series (9, 3, 1, 0.333 ng/ml).

PEG Molecule Plate Preparation

- It is recommended that all PEG standards and samples should be prepared in duplicate.
- Count the strips for the assay and make sure the strips are tightly snapped in the plate frame.
- Leave the unused strips in the foil pouch and store at 2-8 °C. The strips must be stored in the closed foil pouch to prevent moisture because the moisture can damage the PEGylated Molecule Plate.

3. Test Procedure

- Pat the plate on paper towel to remove residual liquid in the wells after wash step.

PEGylated Molecule / Biotin-PEG mAb Incubation

3.1 Add 100 μl of *Assay Diluent* to the NSB (Non-specific binding) wells and 50 μl of a set of prepared PEG standards or samples to the remaining wells separately.

3.2 Add 50 μl of prepared *Biotin-PEG mAb* to each well except the NSB wells.

3.3 Cover the plate with *Plate Sealer* and incubate at 25 °C for 60 minutes.

3.4 Wash the plate with 260 μl of *1 x Wash Solution* for four times.

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Streptavidin-HRP Incubation

3.5 Add 100 μl of *Streptavidin-HRP* to all the wells.

3.6 Cover the plate with *Plate Sealer* and incubate at 37 °C for 10 minutes.

3.7 Wash the plate with 260 μl of *1 x Wash Solution* for four times.

Substrate Reaction and Absorbance Measurement

3.8 Add 100 μl of *TMB Substrate* to all the wells and incubate at 25 °C for 15-20 minutes and protect it from the light.

3.9 Add 50 μl of *Stop Solution* to all the wells to stop the enzyme reaction.

3.10 Read the plate on Microtiter plate reader at 450 nm.

4. Calculation of Data

- To ensure test stability, read the plate at 450 nm immediately after *Stop Solution* addition.
- If the sample is diluted, multiply the interpolated value by the dilution factor to calculate the amount of PEGylated molecule in sample.

4.1 Calculate the average optical density (OD) for each set of replicate wells.

4.2 Calculate the adjusted average OD by subtracting the average NSB OD from the average OD for each standard and sample.

Adjusted average OD = Average OD – Average NSB OD

4.3 Calculate the percent bound for each standard and sample using the following relationship

$$B/B_0\ (\%) = \frac{(\text{Standard or sample OD} - \text{NSB OD})}{(\text{Zero standard OD} - \text{NSB OD})} \times 100$$

4.4 Generate a standard curve by plotting the B/B_0 on the vertical (Y) axis versus the PEGylated molecule standard concentration on the horizontal (X) axis.

4.5 The amount of PEGylated molecule in sample is determined by extrapolating its OD value to the standard curve.

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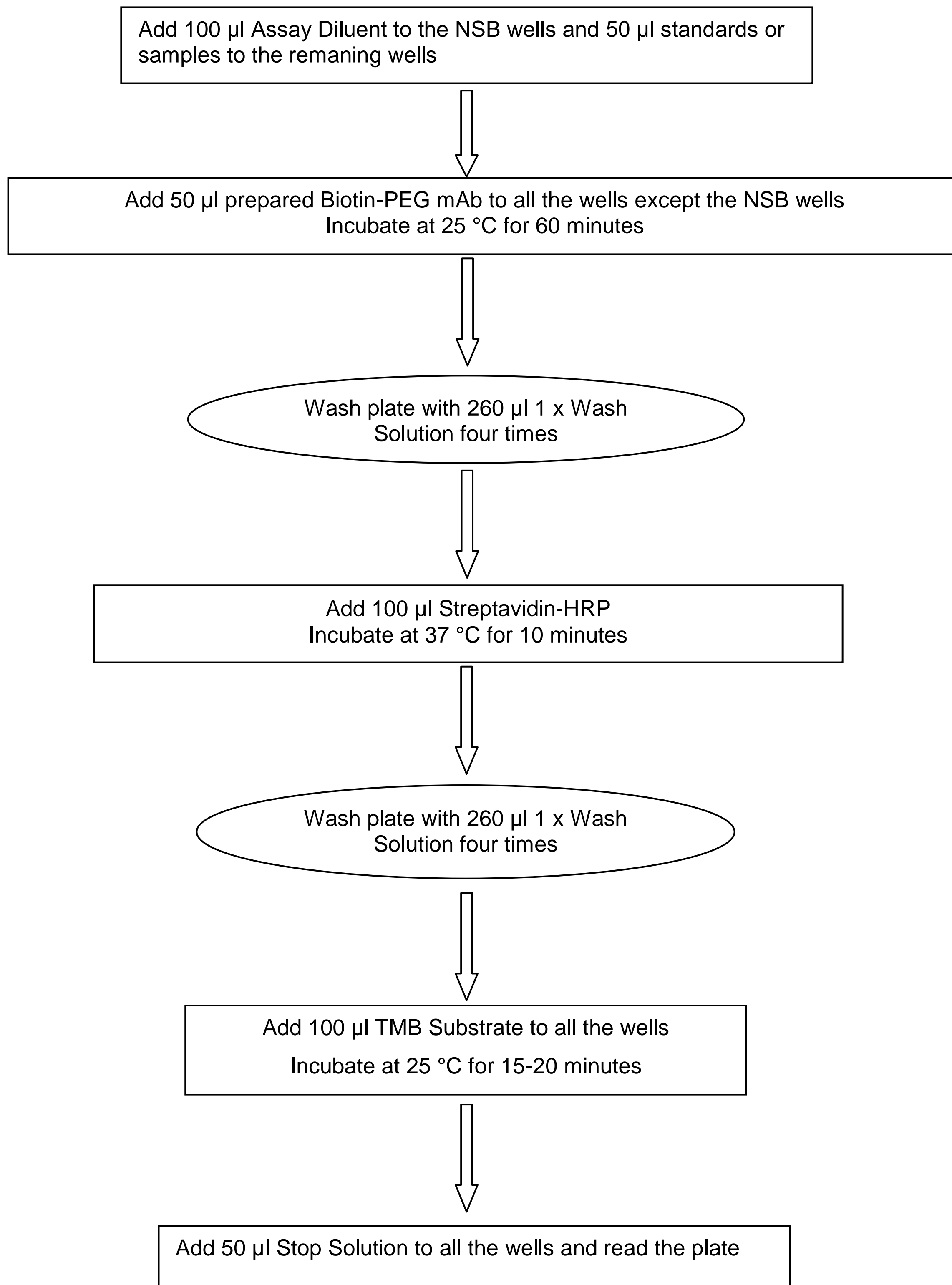
VI. ASSAY PROCEDURE SUMMARY



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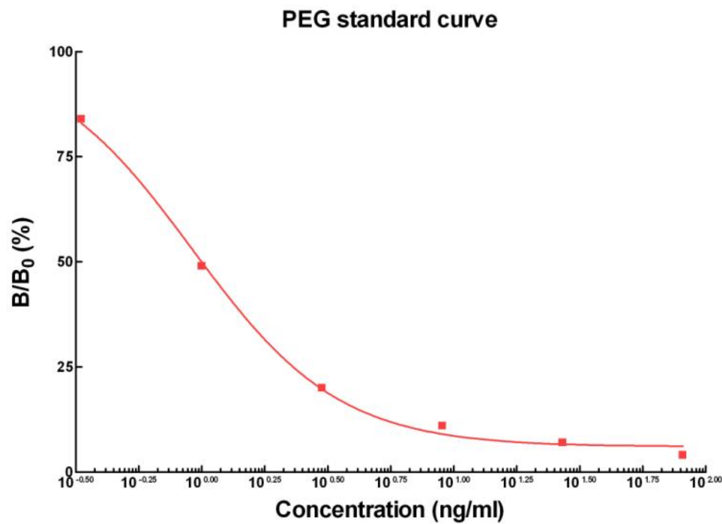
VII. TYPICAL ASSAY DATA

The standard curve below was provided for demonstration only. Operator should use PEGylated molecule of interest to set up standard curve to precisely determine individual PEGylated molecule each time.

PEG Standard (ng/ml)	OD_{450}				$\%B/B_O$
	Duplicate 1	Duplicate 2	Average	Adjusted Average	
NSB	0.059	0.058	0.059	-	-
0	1.995	1.988	1.992	1.933	-
0.333	1.665	1.679	1.672	1.613	84
1	1.030	0.992	1.011	0.952	49
3	0.445	0.437	0.441	0.382	20
9	0.274	0.252	0.263	0.204	11
27	0.197	0.181	0.189	0.130	7
81	0.139	0.130	0.135	0.076	4



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VIII. PRECISION

Intra-assay: Three different levels of PEG40 kDa were tested 20 times on one plate to assess intra-assay precision.
Inter-assay: Three different levels of PEG40 kDa were tested in 20 separate assays to assess inter-assay precision.

Intra-assay				Inter-assay			
# of replicates	Mean (ng/ml)	SD	CV%	# of replicates	Mean (ng/ml)	SD	CV%
20	0.556	0.056	10.1%	20	0.480	0.034	7.0%
20	0.903	0.101	11.2%	20	0.905	0.053	5.8%
20	2.922	0.337	11.5%	20	2.815	0.331	11.8%

IX. SENSITIVITY

The Biotin-PEG mAb in the kit is specific to the backbone of PEG and it is able to bind a broad variety of PEG molecules. Several PEG molecules with different molecular weight have been tested in the assay. The IC50 of each PEG molecule was presented as below.

PEG	IC50 (ng/ml)	PEG	IC50 (ng/ml)
PEG40 kDa	0.947	PEG5 kDa	568.6
PEG30 kDa	0.844	PEG1 kDa	469500
PEG20 kDa	0.861	8arm-PEG-40 kDa	1.3
PEG10 kDa	37.5	4arm-PEG-10 kDa	1150.3

X. RECOVERY

Samples with heat treatment

PEG40 kDa was spiked separately into 1:20 diluted matrices throughout the range of the assay and then were boiled for 5 minutes. The percentage was determined by dividing the calculated value of each spiked sample by the corresponding spiked concentration.

Sample	Spike (ng/ml)	Mean Recovery (%)	Range (%)
Mouse Serum (n=5)	1	95	91-98
	3	106	95-121
	6	95	83-104
Mouse Plasma (n=5)	1	96	90-106
	3	100	94-108
	6	102	89-109
Rat Serum (n=5)	1	101	96-106
	3	93	90-103
	6	86	67-108

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Rat Plasma (n=5)	1	98	95-102
	3	104	103-105
	6	108	94-117
Human Serum (n=5)	1	102	96-111
	3	102	94-109
	6	98	87-106
Human Plasma (n=5)	1	100	94-105
	3	98	96-102
	6	99	86-108

Samples with high dilution

PEG40 kDa was spiked separately into 1:100 diluted matrices throughout the range of the assay without heat treatment. The percentage was determined by dividing the calculated value of each spiked sample by the corresponding spiked concentration.

Sample	Spike (ng/ml)	Mean Recover (%)	Range (%)
Mouse Serum (n=5)	1	98	91-100
	3	96	85-110
	6	91	82-103
Mouse Plasma (n=5)	1	103	97-108
	3	103	95-110
	6	99	89-108
Rat Serum (n=5)	1	101	90-108
	3	99	96-105
	6	98	88-107
Rat Plasma (n=5)	1	99	83-107
	3	97	89-100
	6	98	91-105
Human Serum (n=5)	1	102	98-102
	3	102	92-108
	6	101	88-115
Human Plasma (n=5)	1	103	94-109
	3	107	101-113
	6	96	82-105

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XI. LINEARITY

Samples with heat treatment

PEG40 kDa was spiked into 1:20 diluted matrices at the concentration of 4 ng/ml and then were boiled for 5 minutes. The prepared samples were serially diluted in the Assay Diluent. The percentage was determined by dividing the calculated concentration of each diluted sample by the corresponding spiked concentration.

Dilution		Mouse Serum (n=5)	Mouse Plasma (n=5)
1:2	Average % of Expected	103	107
	Range (%)	99-106	100-115
1:4	Average % of Expected	102	98
	Range (%)	94-108	95-103
1:8	Average % of Expected	96	86
	Range (%)	91-101	83-90

Dilution		Rat Serum (n=5)	Rat Plasma (n=5)
1:2	Average % of Expected	104	102
	Range (%)	96-110	98-109
1:4	Average % of Expected	103	93
	Range (%)	99-109	88-97
1:8	Average % of Expected	96	95
	Range (%)	85-105	90-101

Dilution		Human Serum (n=5)	Human Plasma (n=5)
1:2	Average % of Expected	105	95
	Range (%)	93-114	91-103
1:4	Average % of Expected	103	96
	Range (%)	100-106	89-101
1:8	Average % of Expected	101	87
	Range (%)	91-107	81-93

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Samples with high dilution

PEG40 kDa was spiked into 1:50 diluted matrices at the concentration of 8 ng/ml without heat treatment. The prepared samples were serially diluted in the Assay Diluent. The percentage was determined by dividing the calculated concentration of each diluted sample by the corresponding spiked concentration.

Dilution		Mouse Serum (n=5)	Mouse Plasma (n=5)
1:2	Average % of Expected	89	101
	Range (%)	84-99	95-106
1:4	Average % of Expected	101	108
	Range (%)	92-107	103-118
1:8	Average % of Expected	106	101
	Range (%)	97-112	96-106

Dilution		Rat Serum (n=5)	Rat Plasma (n=5)
1:2	Average % of Expected	100	96
	Range (%)	84-111	90-99
1:4	Average % of Expected	105	104
	Range (%)	89-115	94-112
1:8	Average % of Expected	99	98
	Range (%)	93-106	91-107

Dilution		Human Serum (n=5)	Human Plasma (n=5)
1:2	Average % of Expected	95	99
	Range (%)	85-103	94-102
1:4	Average % of Expected	108	104
	Range (%)	106-110	95-112
1:8	Average % of Expected	109	98
	Range (%)	104-112	96-102

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XII. INTERFERENCE

Some reagents such as Tween, Triton and NP-40 interfere with test results due to structural similarity to PEG. Compatibility of tissue/cell homogenization buffers should be tested before the assay. SDS and RIPA buffer was added into *Assay Diluent* with serial percentage. Same amount of PEG molecules were spiked into the buffers to evaluate reagent interference. The test result demonstrated that RIPA cell lysis buffer should be used below 0.025% (v/v) and SDS is compatible at or below 0.0125% (m/v) in the assay.

Percent (%)	SDS	RIPA
0.1	69.10%	21.90%
0.05	78.20%	28.10%
0.025	90.80%	58.70%
0.0125	97.90%	92.30%
0.00625	99.10%	98.30%
0.003125	101.80%	102.90%
0.001563	105.20%	105.20%

The variety of PEGylated molecules were spiked in Assay Diluent at different concentrations. One set of the samples were treated with heat treatment which was boiled at 100 °C for 5 minutes. The samples with heat treatment and normal samples were assayed in one plate at the same time. The comparison of test results is listed as below which indicates that the heat treatment for the test samples is acceptable in the assay.

PEGylated molecules	ng/ml	No Treatment	Heat Treatment
PEG40 kDa	0	2.260	2.115
	0.333	1.901	1.946
	3	0.529	0.584
	27	0.203	0.216
PEG40 kDa-BSA	0	2.251	2.131
	0.333	1.985	1.942
	3	0.912	0.894
	27	0.283	0.285
PEG40 kDa-IgG	0	2.171	2.115
	0.333	1.927	1.960
	3	0.774	0.761
	27	0.259	0.251
Peginterferon Alfa 2A	0	2.280	2.192
	0.037	1.998	1.973
	0.333	1.263	1.282
	3	0.319	0.317

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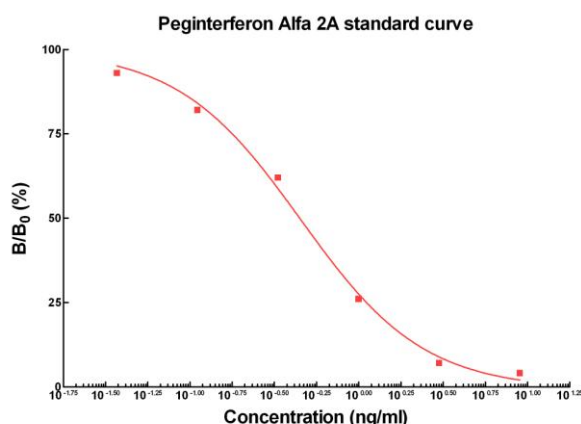
XIII. PHARMACOKINETIC STUDY

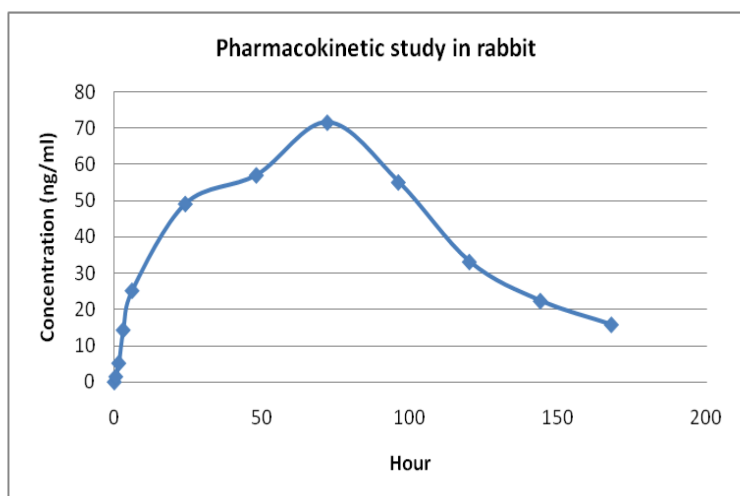
Peginterferon Alfa 2A was used as standard PEGylated molecule to set up standard curve. Peginterferon Alfa 2A is a PEGylated drug. The rabbit was injected with Peginterferon Alfa 2A at the dose of 8.40 µg/kg. The animal blood was collected at different time points. With the standard curve, pharmacokinetic study was performed to measure the PEGylated drug concentration in blood. The standard curve and drug concentration was listed as below.

Peginterferon Alfa 2A standard curve



Peginterferon Alfa 2A (ng/ml)	OD 450			B/Bo (%)
	Duplicate 1	Duplicate 2	Adjusted Average	
NSB	0.056	0.059	0.058	-
0	1.959	1.870	1.857	-
0.037	1.820	1.755	1.730	93
0.111	1.613	1.551	1.524	82
0.333	1.248	1.186	1.159	62
1	0.517	0.558	0.48	26
3	0.176	0.196	0.128	7
9	0.136	0.127	0.074	4

Test Result of Peginterferon Alfa 2A concentration in rabbit blood



Time (h)	Peginterferon Alfa 2A (ng/ml)
0	0
0.5	1.46
1.5	5.18
3	14.33
6	25.20
24	49.14
48	57.05
72	71.58
96	55.10
120	33.13
144	22.35
168	15.79

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XIV.TROUBLESHOOTING

Problem	Probable Cause	Solution
Poor Precision	Wells are not washed or aspirated properly	Make sure the wash apparatus work properly and wells are dry after aspiration
	Wells are scratched with pipette tip or washing needles.	Dispense and aspirate solution into and out of wells with caution
	Particulates are found in the samples	Remove any particulates by centrifugation prior to the assay
	Serum is turbid after heat treatment	Use 30-fold dilution with sample diluent and/or centrifuge the treated sample at high speed
	Hemolysis in blood sample	Prepare new samples
Poor Standard Curve	Improper preparation of standards	Prepare new standards as the manual described
	Wells are not washed or aspirated properly	Make sure the wash apparatus work properly and wells are dry after aspiration
	Pipetting error	Check pipette calibration and repeat assay
	Components are used from other lots or sources	Never substitute any components from another kit
	Components are not brought to room temperature prior to assay	Repeat assay with components that have been equilibrated to room temperature
	Incubation steps are performed at wrong temperatures	Perform incubation step as the manual describes
Weak/No Signal	TMB substrate are not added or added at the wrong time	Follow the manual to add the substrate
	Streptavidin-HRP are not added, or added at the wrong time	Follow the manual to repeat the assay
	Components are used from other lots or sources	Use only lot-specific components
	TMB substrate is contaminated	Use new TMB substrate
	Do not add the proper volumes of reagents	Repeat assay with the required volumes in manual
	Do not incubate the plate for proper time or temperature	Follow the manual to repeat assay
	Do not read the plate immediately after stop solution is added	Read the plate within 30 minutes
High Background	Plate is not washed properly	Make sure the wash apparatus can work properly
	TMB substrate is contaminated	Use new TMB substrate with same Lot
	Evaporation of wells during incubations	Perform incubation steps with plate sealer in repeat assay
	Incorrect incubation times and/or temperatures	Follow the manual to repeat the assay
	TMB substrate is exposed to light	Use new TMB substrate

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XV. RELATED PRODUCTS

- THE™ PEG Antibody, mAb, Mouse A01795-100
- THE™ PEG Antibody [Biotin], mAb, Mouse A01796-100
- Protein A CIP Resin L00433
- Streptavidin-HRP M00091
- Protein A Antibody, mAb, Mouse A01778
- Protein A Antibody [Biotin], mAb, Mouse A01779
- Protein A Antibody, pAb, Chicken A00728
- Protein A Antibody [HRP], pAb, Chicken A00729
- Protein A ELISA Kit L00430

XVI. PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Notes:

- Use this plate layout to record standards and samples assayed.
- The product is used for detection of PEGylated molecules in serum, plasma and other biological samples.

The operator should read technical manual carefully before using this product.

- For research use only. Not for diagnostic use.

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