NCL Method ITA-36

Detection of Naturally Occurring Antibodies to PEG and PEGylated Liposomes

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

Poly(ethylene glycol) (PEG) is commonly used in the pharmaceutical industry to modify recombinant proteins and nanoparticle surfaces to improve hydrophilicity and decrease recognition by the immune system. PEGylated therapeutics and nanoparticles are generally recognized as more stealth than their un-PEGylated counterparts. Despite improved protection from the immune recognition, the immune system is still able to identify these products and mount an antibody response against them. Such immune responses may result in the development of anti-drug antibodies (ADA), and among antibodies specific to biological drug or nanocarrier include the formation of antibodies to the PEG itself. Moreover, several reports suggest the existence of naturally occurring antibodies in the blood of healthy donor volunteers. The physiological significance of anti-PEG antibodies is unknown. However, several studies suggest that they may affect the clearance of PEGylated products (e.g., Accelerated Blood Clearance or ABC phenomenon) and contribute to complement activation and other antibody-mediated toxicities. The purpose of this protocol is to detect the presence of antibodies reactive to PEG2000, mPEG2000 and PEGylated liposomes used for delivery of the anti-cancer drug doxorubicin also known as Doxil. The protocol can be used to assess the presence of both naturally occurring antibodies and antibodies induced as a result of exposure to PEGylated liposomes. The protocol can also be useful in assessing PEG and mPEG antibodies which may react with PEG present in other non-liposomal products.

2. Principles

For the purpose of this protocol PEG2000, mPEG2000 and the PEGylated liposome Doxebo are the antigens (Ags). The Ags are coated on ELISA plates, which after blocking, is incubated with serial dilutions of plasma obtained from donors' or patients' blood. If the antibodies reactive to Ags are present in the tested plasma specimen, they are subsequently detected using anti-human IgG and anti-human IgM conjugated to the enzyme HRP, and visualized by the TMB substrate. The color change in each ELISA well after incubation with the substrate is proportional to the level of antibodies reactive to the Ags coated on the plate. The quantity of antibody is determined in terms of titer, which is the highest dilution of the test plasma demonstrating an optical density reading above the assay threshold. The assay threshold is calculated as a mean OD value of the same plasma sample tested on the plate which was not

coated with the Ag, plus three standard deviations (SD). Five donor plasma samples can be analyzed in one batch, and four plates are required for each batch.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of suppliers listed below; their inclusion is for informational purpose only. Equivalent supplies from alternative vendors can be substituted.

3.1 Reagents

1. PEG 2000 (Sigma, 821037)
2. mPEG2000 (Laysan Bio, MPEG-SH-2000-1g)
3. Doxil liposome set, need 2 sets (Avanti Lipids, 300107)
4. Instant Nonfat Dry Milk.
5. Mouse Anti-PEG monoclonal IgM Antibody (ANPEG-1) (500 µg) (ANTP, 90-1010-500UG)
6. Biotinylated Anti-PEG monoclonal IgM Antibody (ANPEG-1) (100 µg) (ANTP, 90-1052-100UG)
7. Donkey anti-human IgG-HRP, 0.5mL (Jackson ImmunoResearch, 709-035-149)
8. Rabbit anti-human IgM-HRP (Jackson ImmunoResearch Lab, 309-035-095)
9. Goat anti rat IgM-HRP conjugated (Jackson ImmunoResearch, 112-035-075)
10. Donkey anti-rabbit IgG HRP conjugated, 0.5 mL (Jackson ImmunoResearch Lab, 711-035-152)
11. Rabbit anti PEG IgG (Abcam, 51257)
12. PBS (10x), 1L (HyClone, SH30258.02)
13. Fetal Bovine Serum (FBS), 500 mL (HyClone, SH30070.03)
14. BupH Carbonate-Bicarbonate Buffer Packs (Pierce, 28382)
15. Ultra TMB-ELISA Substrate (Pierce, 34028)
16. AGP6 rat anti-PEG antibody (Taiwan)
17. CHAPS, PlusOne, 1g (GE Healthcare Life Sciences, 17-1314-01)

3.2 Materials

1. Nunc Maxisorb 96-well ELISA plates (Thermo, 442404)\
2. Pipettes, 0.05 to 10 mL
3. Paper towels

4. Polypropylene tubes, 15 and 50 mL
5. Plate sealers

3.3 Equipment

1. Microcentrifuge
2. Refrigerator, 2-8°C
3. Freezer, -20°C
4. Vortex
5. Plate washer
6. Plate reader capable of measuring optical density at 450 nm

4. Reagent and Control Preparation

4.1 Coating Buffer (BupH Carbonate-Bicarbonate)

Dissolve one pack of BupH Carbonate-Bicarbonate in 500 mL distilled water and mix well. This step produces 0.2 M carbonate-bicarbonate buffer with pH 9.4. Filter through 0.2 µm filter and store at room temperature for up to one month.

4.2 Wash Buffer (1X PBS + 0.1% CHAPS)

Add 100 mL of 10X PBS to 900 mL distilled water. Add 1 g CHAPS and mix well. Store at room temperature for one month.

4.3 Blocking Buffer (5% Nonfat Dry Milk in 1X PBS)

Weigh 25 g Nonfat Dry Milk and dissolve in 500 mL of 1X PBS and mix well. Store at 4°C.

4.4 Dilution Buffer A (2% Nonfat Dry Milk in 1X PBS)

Weigh 10 g Nonfat Dry Milk and dissolve in 500 mL 1X PBS and mix well. Store at 4°C.

4.5 Dilution Buffer B (4% FBS + 2% Nonfat Dry Milk in 1X PBS)

Add 20 mL of FBS to 480 mL of Dilution Buffer A. Store at 4°C.

4.6 Antigens

a. Dilute PEG2000 in blocking buffer to a final concentration of 10 µg/mL.
b. Dilute mPEG2000 in blocking buffer to a final concentration of 10 µg/mL.
c. Dilute Doxebo stock 300 times in locking buffer. This dilution brings the mPEG concentration in the sample to 11 µg/mL.

4.7 Positive Control IgG

Dilute rabbit anti-PEG IgG (PEG-B-47) in pooled normal human serum or plasma to a final concentration 10 µg/mL. This antibody will not recognize PEG2000 because they are specific to the methoxy group, according to the manufacturer's info.

4.8 Positive Control IgM

Dilute rat anti-PEG IgM (AGP6) in pooled normal human serum or plasma to a final concentration 10 µg/mL. This antibody should recognize all PEGs because, according to the producer's info, they are specific to PEG backbone.

4.9 Stop Solution, 2 N sulfuric acid (H_2SO_4)

Slowly add 27.7 mL H_2SO_4 into 200 mL of dH_2O water, mix the solution thoroughly, let it cool and bring the solution to 500mL with dH_2O using a 1000 mL graduated cylinder. Mix well and store in a bottle at room temperature.

5. Procedure

5.1 Coat 3 ELISA plates with 125 µL/well of 1 µg/mL of ANPEG-1 antibody in BupH buffer overnight at 4°C. For the uncoated plate, add 125 µL/well of plain BupH buffer and incubate overnight at 4°C.

Note: There are two categories of plates. Category A is used to assess the levels of PEG-specific IgG, and Category B is used for the detection of the anti-PEG IgM. You will need 1 uncoated and 3 plates coated with individual antigen for each category. Refer to Appendix for example plate maps.

5.2 Aspirate and discard coating solution, tap the plate dry on paper towels, and add 250 µL/well of blocking buffer. Incubate 1 hr at room temperature (RT). (Uncoated plate incubates with blocking buffer 2 hr at RT.)

5.3 Aspirate blocking buffer from coated plates and tap the plates dry on paper towels.

5.4 Add 125 µL/well of Antigen to coated plates and incubate at RT for 1 hr.

Note: There are 3 antigens total (PEG2000, mPEG2000 and Doxebo). For each group of 5 donors, prepare 4 plates in step 1 (3 coated with ANPEG-1 and 1 uncoated).

5.5 Wash plates once with 1X PBS (300 µL/well).

5.6 Add 240 µL of Dilution Buffer A to wells in row A. Add 125 µL of Dilution Buffer B to wells in rows B-H.

5.7 Add 10 µL (initial 1:25 dilution) of the test serum and controls to corresponding wells in row A.

5.8 Using multichannel pipette, transfer 125 µL from row A to row B. Pipet up and down several times and transfer 125 µL from row B to row C. Repeat mixing and transferring to the next row until row H. After mixing, collect 125 µL from row H and discard it. This step results in serial 2-fold dilutions of the test sera.

5.9 Incubate the plate at RT for 1 hr.

5.10 Wash plate 2 times with wash buffer. Rotate the plate after the first wash. Buffer volume per well is 300 µL.

5.11 Wash plate 1 time with 1X PBS (300 µL/well). Invert and tap dry on paper towel.

5.12 Add 125 µL per well of conjugate and incubate at RT for 1 hr.

***Important**: IgG plates will receive anti-human IgG-HRP. IgM plates will receive anti-human IgM-HRP. Wells containing positive control IgG serum will receive anti-rabbit IgG-HRP. Wells containing positive control IgM serum will receive anti-rat-IgM-HRP.*

5.13 Repeat washes in steps 5.11 and 5.12. Buffer volume per well is 300 µL.

5.14 Add 125 µL of substrate to each well and develop the plate for 10-30 min.

5.15 Add 30 µL of stop solution, tap the plate to mix and read the absorbance at 450 nm.

5.16 The antibody titer is determined by selecting the highest dilution of the test sample which generated an OD signal on the antigen-coated plate 2-fold higher than the mean background signal determined for the same sample on the uncoated plate.

6. Appendix.

Example of Plate Maps

Category A – IgG

Uncoated *(this plate receives the same treatments as coated plates except for the initial coating with ANPEG-1 antibody)*

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
C	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
D	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
E	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
F	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
G	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
H	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5

From row A to H the titers are: 25, 50, 100, 200, 400, 800, 1600, 3200. **Columns 1 and 2 receive donkey anti-rabbit-IgG HRP. All other wells receive donkey anti-human IgG-HRP.**

PEG 2000-coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
C	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
D	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
E	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
F	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
G	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
H	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5

From row A to H the titers are: 25, 50, 100, 200, 400, 800, 1600, 3200. **Columns 1 and 2 receive donkey anti-rabbit-IgG HRP. All other wells receive donkey anti-human IgG-HRP**

mPEG-2000-Coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
C	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
D	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
E	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
F	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
G	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
H	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5

From row A to H the titers are: 25, 50, 100, 200, 400, 800, 1600, 3200. **Columns 1 and 2 receive donkey anti-rabbit-IgG HRP. All other wells receive donkey anti-human IgG-HRP**

Doxebo-Coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
C	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
D	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
E	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
F	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
G	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
H	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5

From row A to H the titers are: 25, 50, 100, 200, 400, 800, 1600, 3200. **Columns 1 and 2 receive donkey anti-rabbit-IgG HRP. All other wells receive donkey anti-human IgG-HRP**

Category B – IgM

Uncoated *(this plate receives the same treatments as coated plate except for the initial coating with ANPEG-1 antibody)*

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
C	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
D	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
E	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
F	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
G	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
H	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5

From row A to H the titers are: 25, 50, 100, 200, 400, 800, 1600, 3200. **Columns 1 and 2 receive goat anti-rat IgM-HRP. All other wells get rabbit anti-human IgM-HRP.**

PEG 2000-Coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
C	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
D	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
E	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
F	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
G	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
H	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5

From row A to H the titers are: 25, 50, 100, 200, 400, 800, 1600, 3200. **Columns 1 and 2 receive goat anti-rat IgM-HRP. All other wells get rabbit anti-human IgM-HRP.**

mPEG 2000-Coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
C	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
D	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
E	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
F	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
G	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
H	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5

From row A to H the titers are: 25, 50, 100, 200, 400, 800, 1600, 3200. **Columns 1 and 2 receive goat anti-rat IgM-HRP. All other wells get rabbit anti-human IgM-HRP.**

Doxebo-Coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
C	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
D	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
E	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
F	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
G	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
H	PC	PC	TS 1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5

From row A to H the titers are: 25, 50, 100, 200, 400, 800, 1600, 3200. **Columns 1 and 2 receive goat anti-rat IgM-HRP. All other wells get rabbit anti-human IgM-HRP.**