



## **NCL Method ITA-36**

### **Detection of naturally occurring antibodies to PEG and PEGylated liposomes in plasma of human donor volunteers**

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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 surface to improve hydrophilicity and decrease their 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 response may result in the development of anti-drug antibody (ADA), and among antibodies specific to biological drug or nanocarrier include the formation of antibodies to the PEG itself. Moreover, several reports suggested the existence of naturally occurring antibodies in the blood of healthy donor volunteers. The physiological significance of the anti-PEG antibodies is unknown. However, several studies suggested that they may affect the clearance of PEGylated products (e.g., Accelerated Blood Clearance or ABC phenomenon) and contribute to the 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 anti-cancer drug doxorubicin also known as Doxil formulation. The protocol can be used to assess the presence of both the naturally occurring antibodies and the 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 PEGylated liposome Doxebo are the antigens (Ags). The Ags are coated on ELISA plates, which after blocking any unbound sites, are incubated with serial dilutions of the plasma obtained from donor's or patient's 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 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 the 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 not coated with the Ag plus 3 standard deviations (SD). Five donor plasma samples can be analyzed in one batch, and four plates are required for each batch.

### 3. Reagents

*Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.*

1. PEG 2000, Sigma, <http://www.sigmaaldrich.com/catalog/product/mm/821037?lang=en&region=US>
2. mPEG2000, Laysanbio, [http://www.laysanbio.com/index.php?src=directory&view=products&submenu=Products&category=M-SH-2K&query=category.eq.M-SH-2K&refno=318&srctype=products\\_detail](http://www.laysanbio.com/index.php?src=directory&view=products&submenu=Products&category=M-SH-2K&query=category.eq.M-SH-2K&refno=318&srctype=products_detail)
3. Doxil liposome set, Avanti Lipids, need 2 sets <https://avantilipids.com/product/300107/>
4. Instant Nonfat Dry Milk.
5. mouse Anti-PEG monoclonal IgM Antibody (ANPEG-1) (500 µg), ANTP, Product No. 90-1010-500UG, <http://www.anptinc.com/anti-peg-monoclonal-igm-antibody>
6. Biotinylated Anti-PEG monoclonal IgM Antibody (ANPEG-1) (100 µg), ANTP, Product No. 90-1052-100UG, (<http://www.anptinc.com/biotinylated-anti-peg-antibody>)
7. Donkey anti-human IgG-HRP, Jackson ImmunoResearch, <https://www.jacksonimmuno.com/catalog/products/709-035-149> 0.5mL
8. Rabbit anti-human IgM-HRP, Jackson ImmunoResearch Lab <https://www.jacksonimmuno.com/catalog/products/309-035-095>
9. Goat anti rat IgM-HRP conjugated, Jackson ImmunoResearch , <https://www.jacksonimmuno.com/catalog/products/112-035-075>
10. Donkey anti-rabbit IgG HRP conjugated, Jackson ImmunoResearch Lab <https://www.jacksonimmuno.com/catalog/products/711-035-152> need 0.5mL
11. Rabbit anti PEG IgG, Abcam, cat#51257, <http://www.abcam.com/polyethylene-glycol-antibody-peg-b-47-ab51257.html>
12. PBS (10x), 1L (Hyclone, SH30258.02)
13. Fetal Bovine Serum (FBS), 500ml (Hyclone SH30070.03)
14. BupH Carbonate-Bicarbonate Buffer Packs (Pierce, 28382)
15. Ultra TMB-ELISA Substrate (Pierce, 34028)

16. Nunc Maxisorb 96 well ELISA plates (Thermo, 442404)
17. AGP6 rat anti-PEG antibody (Taiwan)
18. CHAPS, PlusOne, 1g (GE Healthcare Life Sciences, 17-1314-01)

#### **4. Buffers and controls**

##### **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 pH9.4. Filter through 0.2  $\mu$ m filter and store at room temperature for up to one month.

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

Add 100 mL of 10 x PBS to 900 mL distilled water. Then 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**

1. Dilute PEG2000 in blocking buffer to a final concentration of 10  $\mu$ g/mL.
2. Dilute mPEG2000 in blocking buffer to a final concentration of 10  $\mu$ g/mL.
3. Dilute Doxibo stock 300 times in blocking buffer. This dilution brings mPEG concentration in the sample to 11  $\mu$ g/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<sub>2</sub>SO<sub>4</sub>)

Slowly add 27.7 mL H<sub>2</sub>SO<sub>4</sub> into 200mL of dH<sub>2</sub>O water, mix the solution thoroughly, let it cool and bring the solution to 500mL with dH<sub>2</sub>O using a 1000 mL graduated cylinder. Mix well and store in a bottle at room temperature.

### 5. Procedure

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 125µL/well of plain BupH buffer to it and incubate overnight at 4°C.

*Note: There are two categories of the 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 the example of plate maps.*

2. Aspirate and discard coating solution, tap the plate dry on paper towels and add 250 µL/well of blocking buffer. Incubate 1h at RT (uncoated plate incubates with blocking buffer 2h at RT)
3. Aspirate blocking buffer from coated plates and tap the plates dry on paper towels.
4. Add 125 µL/well of Antigen to coated plates and incubate at RT for 1h.  
*Note: There are 3 antigens total (PEG2000, mPEG2000 and Doxibo). For each group of 5 donors, prepare 4 plates in step 1 (3 coated with ANPEG-1 and 1 uncoated).*
5. Wash plates once with 1X PBS (300ul/well).
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.
7. Add 10 µL (initial 1:25 dilution) of the test serum and controls to corresponding wells in row A.
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 dilution of the test sera.
9. Incubate the plate at RT for 1 h.
10. Wash plate 2 times with wash buffer. Rotate the plate after the first wash. Buffer volume per well is 300 µL.

11. Wash plate 1 time with 1X PBS (300  $\mu$ L/well). Invert and tap dry on paper towel.
12. Add 125  $\mu$ L per well of conjugate and incubate at RT for 1h.  
***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*
13. Repeat washes in steps 11 and 12. Buffer volume per well is 300  $\mu$ L
14. Add 125  $\mu$ L of substrate to each well and develop the plate for 10-30 min.
15. Add 30  $\mu$ L of stop solution, tap the plate to mix and read the absorbance at 450 nm

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**

**Category A – IgG (continues...)**



### 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.**

#### Category B – IgM (continues...)

#### 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.**

#### Doxibo-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.**