NCL Method ITA-5.2

Analysis of Complement Activation by Single-Plex EIA or Multiplex ELISA

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Protocol adapted from:

Neun BW, Ilinskaya AN, Dobrovolskaia MA. *Analysis of Complement Activation by Nanoparticles*, in *Characterization of nanoparticles intended for drug delivery*, S. McNeil, Editor. Methods in Molecular Biology. Vol. 1628, **2018**, Humana Press, New York, NY. p. 149-160. doi: 10.1007/978-1-4939-7352-1_13

Please cite this protocol as:

Neun B, Cedrone E, Dobrovolskaia MA, NCL Method ITA-5.2: Analysis of Complement Activation by single-plex EIA or multiplex ELISA. https://ncl.cancer.gov/resources/assay-cascade-protocols DOI: 10.17917/EQCT-3C51

1. Introduction

This document describes a protocol for quantitative determination of complement activation by an Enzyme Immunoassay (EIA). The complement system represents an innate arm of immune defense and is named so because it "complements" the antibody-mediated immune response. Three major pathways leading to complement activation have been described: they are the classical pathway, alternative pathway and lectin pathway (Figure 1). The classical pathway is activated by immune (antigen-antibody) complexes. Activation of the alternative pathway is antibody independent. The lectin pathway is initiated by plasma protein mannose-binding lectin.

The complement system is a group of ~30 protein that includes several components (C1 - C9), and Factors (B, D, H, I, and P). Activation of any of the three pathways results in cleavage of the C3 component of the complement system [1, 2].

This protocol is intended for follow-up studies on samples which demonstrated a positive response in the qualitative assay (NCL Method ITA 5.1). It can also be performed as an independent protocol when high throughput analysis is needed.

2. Principles

In the protocol presented herein, human plasma is exposed to a test material and subsequently analyzed by EIA for the presence of the complement components C4d, iC3b and Bb. The antibodies specific to these proteins are immobilized on 96 well plates and are obtained from commercial suppliers. Test nanoparticles found to be positive in the qualitative western blot assay are then subject to a more detailed investigation aimed at delineation of the specific complement activation pathway. Detection of elevated levels of C4d protein is indicative of complement activation via the classical or lectin pathway. Elevation in Bb levels is a sign of alternative pathway activation. Estimation of iC3b levels is used to confirm, in a more accurate, quantitative way, the results of the initial western blot screen specific to the C3 component of the complement system.

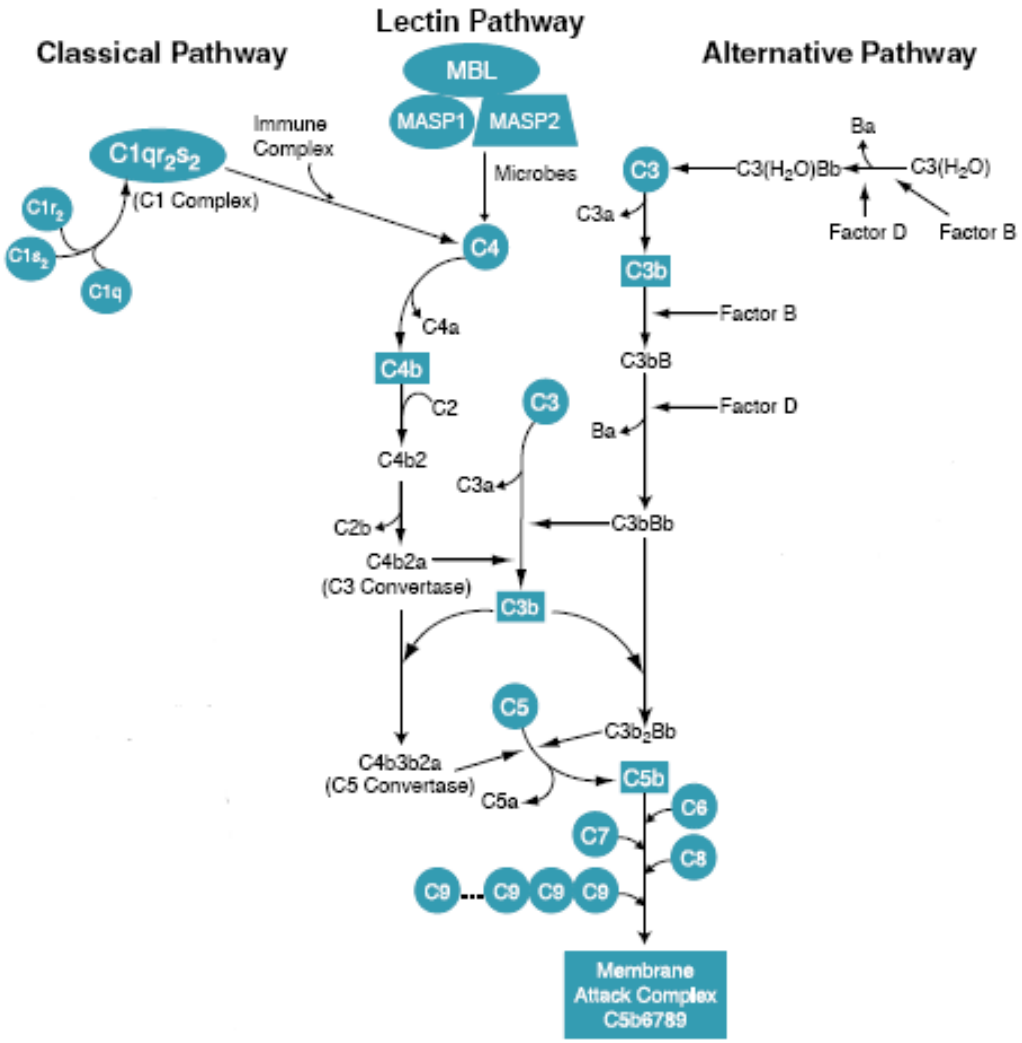

Figure 1. Complement activation pathways. (This illustration is reproduced from reference 1 with permission from EMD Biosciences, Inc.)

3. Reagents, Materials, and Equipment

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.

3.1 Reagents

3.1.1 Sterile Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)

3.1.2 Cobra Venom Factor (positive control) (Quidel Corp., A600)

3.1.3 Veronal Buffer (Boston BioProducts, IBB-260)

3.1.4 Pooled human plasma, anti-coagulated with Sodium citrate

3.1.5 MicroVue iC3b EIA kit (Quidel Corp., A006)

3.1.6 MicroVue C4d fragment EIA kit (Quidel Corp., A0008)

3.1.7 MicroVue Bb Plus EIA kit (Quidel Corp., A027)

3.1.8 MicroVue Complement Multiplex (8-plex) EIA Kit (Quidel Corp., A900)

3.1.9 Doxil (Doxorubicin HCl, liposome, injection) *This is a prescription medication available from a licensed pharmacy and may not be available to some research laboratories.*

3.1.10 Cremophor, (Sigma, C 5135)

3.1.11 Complement activator (Quidel, https://www.quidel.com/research/complement-reagents/complement-activator)

3.1.12 Taxol (Paclitaxel in Cremophor EL) *This is a prescription medication available from a licensed pharmacy and may not be available to some research laboratories.*

3.2 Materials

3.2.1 Pipettes covering the range from 0.05 to 1 mL

3.2.2 Microcentrifuge tubes, 1.5 mL

3.2.3 Pipet tips, 0.5 µL – 1.0 mL

3.2.4 Multichannel (8-12 channel) pipettor with volumes 50-300 µL

3.2.5 15 and 50 mL conical tubes

3.2.6 Reagent reservoirs

3.3 Equipment

3.3.1 Microcentrifuge

3.3.2 Centrifuge capable of running at 2500xg, with a swinging basket set up to hold 5 cc vacutainer tubes

3.3.3 Refrigerator, 2-8˚C

3.3.4 Freezer, -20˚C

3.3.5 Vortex

3.3.6 Incubator, 37˚C

3.3.7 ELISA plate reader (for single plex) capable of operating at 405 nm

3.3.8 Q-View Imager Pro (for multi-plex) or similar imaging system

4. Reagent and Control Preparation

4.1 Positive Control 1 (Traditional substance known to activate complement)

4.1.1 *Cobra Venom Factor (CVF)* is supplied frozen solution. Thaw this stock, prepare single use aliquots and store them at a nominal temperature of -80˚C for as long as performance is acceptable. Avoid repeated freeze/thaw cycles. After thawing single use aliquot and using it in the assay, discard any leftover material. For this experiment, use 30 µL (1.1-50 U) of CVF solution. This control activates complement system through alternative pathway.

4.1.2. *Heat Aggregated Gamma Globulin (HAGG*) acts similarly to naturally occurring immune complexes and is very potent activator of complement through the classical pathway. This control is available from Quidel under name "Complement Activator". Handling and storage are according to the manufacturer's instructions. Avoid repeated freeze/thaw cycles when stored at -20 °C.

4.2 Positive Control 2 (nanoparticle relevant)

4.2.1 Cremophor-EL

Cremophor-EL is an excipient commonly used in the pharmaceutical industry to dissolve hydrophobic drugs. Cremophor-EL is a nanosized micelle which is known to induce complement activation related pseudo allergy (CARPA) syndrome [2], and therefore is used as a nanoparticle relevant control. The following procedure can be used to prepare Cremophor-EL with a composition that is similar to the clinical formulation of Paclitaxel (Taxol) (527 mg of purified Cremophor EL* (polyoxyethylated castor oil) and 49.7% (v/v) dehydrated alcohol, USP and 2 mg of citric acid per 1 mL). Store at room temperature.

To prepare Cremophor-EL, mix commercial Cremophor 1:1 with ethanol containing 2 mg/mL of citric acid to mimic the concentration of Cremophor-EL, citric acid, and ethanol used in Taxol and the generic formulation of paclitaxel.

4.2.2 Cremophor-EL Formulated Paclitaxel (Taxol)

Taxol can be used as an alternative nanoparticle relevant positive control. It is supplied at a stock concentration 6 mg/mL of paclitaxel. When used in this assay, the final concentration of paclitaxel is 2 mg/mL. Store 2-8ºC.

4.2.3 PEGylated Liposomal Doxorubicin (Doxil)

Doxil can also be used as nanoparticle relevant positive control [3]. Doxil is doxorubicin formulated in nanoliposomes. It is available through the pharmacy as 20 mg of Doxorubicin HCl in 10 mL vehicle. Store 2-8ºC.

4.3 Inhibition/Enhancement Control (IEC)

Use positive control sample after the incubation. Prior to loading this sample onto ELISA plate, add nanoparticles at the same final concentrations as in the study samples. For example, one can mix 20 µL of the positive control sample and 10 µL of the test nanoparticle. The test result for this sample needs to be adjusted by the dilution factor 1.5 prior to comparison of the test value to the test value of the positive control sample. If the test results are different by no more than 25%, the test nanoparticle at the given concentration does not interfere with detection of the complement split product by ELISA.

4.4 Negative Control (PBS)

Sterile Ca^{2+}/Mg^{2+}-free PBS is used as a negative control. Store at room temperature for up to 6 months.

4.5 Vehicle Control (relevant to each given nanoparticle)

When nanoparticles are not formulated in saline or PBS, the vehicle sample should be tested to estimate the effect of excipients on the complement system. This control is specific to each given nanoparticle sample. It should be prepared to match the formulation buffer of the nanoparticle by both composition and concentration.

4.5 Stop Solution (HCl)

Stop solution is provided with each kit but can also be prepared separately. Dilute stock hydrochloric acid to a final concentration of 1.0 N. Filter and store and room temperature for up to 2 weeks.

5. Preparation of Study Samples

This assay requires 400 μL of nanoparticles in PBS at a concentration 3 times higher than the highest final tested concentration. The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called "theoretical plasma concentration". Considerations for estimating theoretical plasma concentration were reviewed elsewhere [4] and are summarized in Box 1 below.

The assay will evaluate 4 concentrations: 10X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, theoretical plasma concentration and two 1:5 serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration.

For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 6 mg/mL will be prepared and diluted 10-fold (0.6 mg/mL), followed by two 1:5 serial dilutions (0.12 and 0.024 mg/mL). When 0.1 mL of each of these samples is added to the test tube and mixed with 0.1 mL of plasma and 0.1 mL of veronal buffer, the final nanoparticle concentrations tested in the assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL.

Box 1. Example Calculation to Determine Nanoparticle Concentration for In Vitro Tests

In this example, we assume a mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$Human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\ mg/kg}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in blood, which is used as the in vitro test concentration.

$$in\ vitro\ concentration_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg * 10\ mg/kg}{5.6\ L} = 0.125\ mg/mL$$

6. Plasma Collection and Storage

Blood is drawn into vacutainer tubes containing anticoagulant. Sodium citrate is ideal anti-coagulant for this assay, however depending on phlebotomy paraphernalia, plasma anti-coagulated with sodium citrate may result in high background in the ELISA assay. In this case using K_2EDTA as the anticoagulant is acceptable. The first 5-10 mL of blood should be discarded and not used to prepare plasma. For optimal results, it is important to keep blood at 20-24ºC, to avoid exposure to high temperatures (summertime) and low temperatures (wintertime), and to avoid prolonged (> 1 hr) storage. Blood is transported to the lab in a contained Styrofoam box with warm packs (20-24°C). To prepare plasma, the blood is spun down in a centrifuge 10 minutes at 2500xg. Plasma is evaluated for the presence of hemolysis. Discolored plasma (an indication of hemolysis) is not used to prepare the pool. Individual plasma specimens that did not show any indication of hemolysis are pooled and mixed in a conical tube. Plasma must be used for complement testing within 1 hour after collection. Pooled plasma can be used and prepared by mixing plasma from at least 2 individual donors. The assay can also be performed in the plasma from individual donors. In this case analyze plasma from at least 3 donors.

It is possible to use pooled sodium citrate plasma from commercial suppliers, however, when placing the order, one needs to notify the supplier that the plasma is intended for complement testing so no delays between blood draw and plasma collection occurs. The supplier then freezes

the plasma immediately after collection and ships it to the lab on dry ice. When using frozen plasma for the complement activation assay, it is important to avoid repeated freeze/thaw cycles. The frozen plasma should be thawed in a water bath containing ambient tap water, mixed gently and used immediately after thawing. It is also advised to avoid indefinite storage of frozen plasma at -20ºC. The sooner the frozen plasma is used, the better the results are. In general, the degree of complement activation estimated by comparing intensity of the C3 split product in the positive control with that of the negative control is greater in fresh plasma than in thawed plasma.

7. Experimental Procedure for Sample Preparation

7.1 In a microcentrifuge tube, combine equal volumes (100 µL of each) of veronal buffer, human plasma, and a test-sample (i.e., positive control, negative control, nanoparticles, or vehicle control if different than PBS). Prepare two replicates of each sample.

7.2 Vortex tubes to mix all reaction components, spin briefly in a microcentrifuge to bring any drops down, and incubate in an incubator at a nominal temperature of 37˚C for 30 minutes.

7.3 Prepare 100 µL aliquots and either use in EIA immediately or freeze at -20ºC for later analysis.

8. Experimental Procedure for Single-Plex EIA

8.1 Follow the manufacturer's instructions to reconstitute complement standard, wash buffers and controls.

8.2 Dilute plasma samples prepared in step 7.3 in complement specimen diluent reagent (provided with each kit). Use the following dilution guide for each individual assay:

iC3b – 1:1500 for positive control sample; 1:30 for negative control and other test samples

C4d – 1:30 for all samples

Bb – 1:75 for all samples

Note: The dilution factors should be determined by each laboratory and adjusted if needed.

8.3 Follow manufacturer's instructions for plate loading volumes, incubation time, and plate washing.

8.4 Read plate on a plate reader at 405 nm.

9. Experimental Procedure for Multi-plex ELISA

9.1 Follow manufacturer's instructions to reconstitute standards, wash buffer, and controls.

9.2 Dilute plasma samples prepared in step 7.3 with sample diluent (provided with each kit). PC, NC, and all samples are diluted 1:100.

9.3 Follow manufacturer's instructions for loading volumes, incubation time, and plate washing.

9.4 Capture a 300-second image of the plate on the Q-View Imager Pro or a 270-second image on the Q-View Imager LS.

10. Data Analysis

Do not forget to use the appropriate dilution factor for control and study samples. Compare determined amount of complement components between positive control or study samples with that in the negative control. An increase in the complement component species 2.0-fold or higher above the background (negative control) constitutes a positive response. If a nanoparticle under study generated a positive response in any of the EIA assays, compare the degree of activation between this particle and the Doxil or other nanoparticle-relevant control. Doxil is used in the clinic and is known to induce complement activation related hypersensitivity reactions in sensitive patients [5]. Using Doxil helps to interpret results of this *in vitro* study for a test nanoparticle. If the degree of activation observed for the test nanoparticle is equal to or greater than that observed for Doxil, this nanoparticle formulation will most likely cause similar or stronger hypersensitivity reactions in patients and may require modifications before entering *in vivo* preclinical and clinical phases. If the degree of activation is lower than that of Doxil, complement activation should be considered when designing the *in vivo* evaluation phase for the given particle, but it is less likely to cause concerns similar to Doxil.

11. Acceptance Criteria

11.1 Percent CV between replicates of standard curve, quality controls, and test samples should be within 25%.

11.2. Percent difference from theoretical for each of the standard curve samples should be within 25%, and correlation coefficient should be at or above 0.98.

11.3 Run is acceptable if conditions described in 11.1 and 11.2 are met.

11.4 The degree of complement activation in the positive control sample, estimated by comparing levels of individual complement split product in the positive control with that in the negative controls should be at or above 2.0-fold.

Note: Cobra venom factor activates complement through the alternative assay, so this control will not provide a positive response in the C4d assay. HAGG is a positive control for C4d assay. Doxil is positive in the C4d EIA.

12. References

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13. Abbreviations

CVF	cobra venom factor
PBS	phosphate buffered saline
EIA	enzyme immunoassay
HAGG	heat aggregated gamma globulin
HRP	horseradish peroxidase
IEC	inhibition/enhancement control
IgG (H + L)	immunoglobulin G (high and low chains)
NC	negative control
PC	positive control