NCL Method ITA-5.2

Analysis of Complement Activation by EIA

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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

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 the plasma protein mannose-binding lectin.

The complement system is composed of several components (C1, C2.....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].

2. Principles

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. Detection of elevated levels of C4d protein is indicative of complement activation via the classical or lectin pathway. Elevation of 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.

This protocol is intended for follow-up studies on samples that demonstrated a positive response in the qualitative assay (NCL method ITA 5.1). Test nanoparticles found to be positive in the qualitative western blot assay can be subjected to this more detailed investigation aimed at delineation of the specific complement activation pathway. Alternatively, this assay can also be performed as an independent protocol when high throughput analysis is needed.

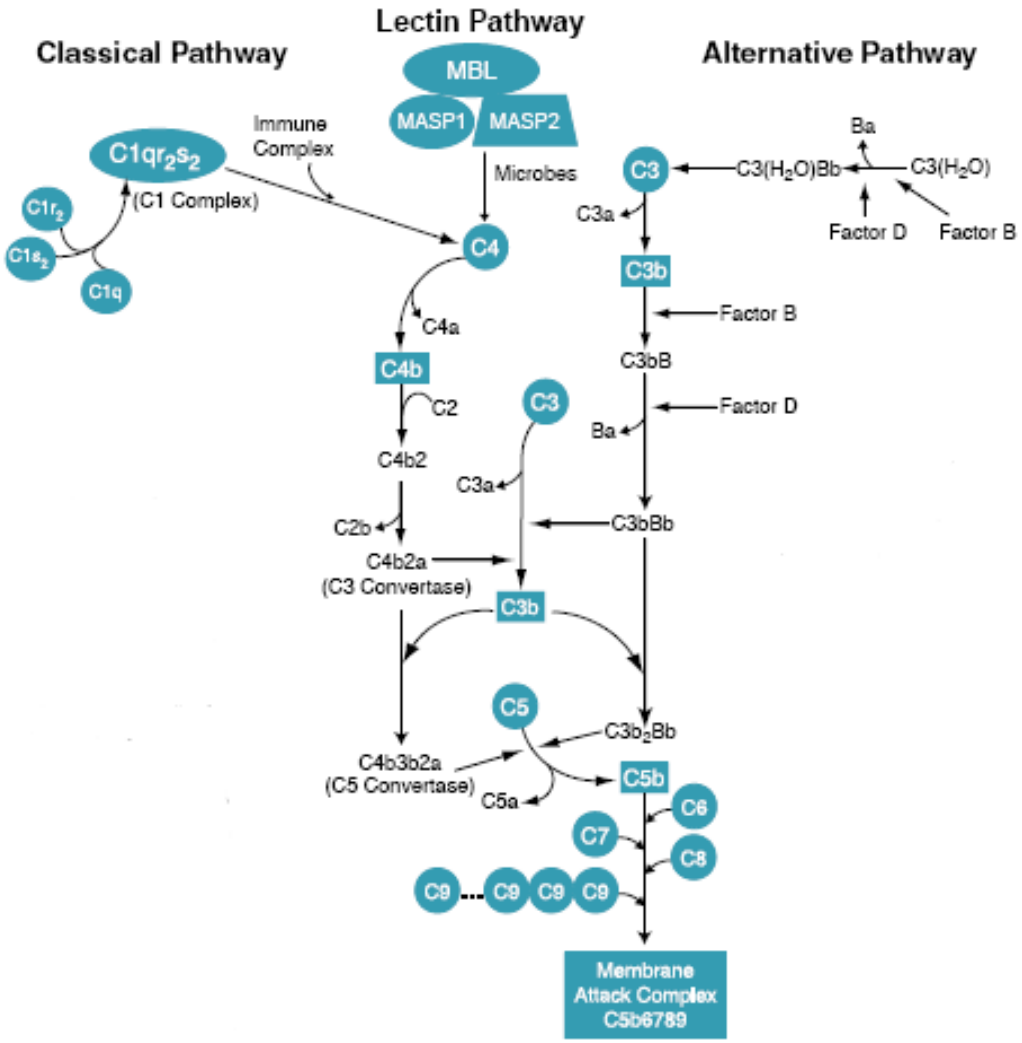

Figure 1. Complement activation pathways. (This illustration is reproduced from reference 1 with permission from EMD Biosciences, Inc.)

3. Reagents, Materials, and Equipment

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

3.1 Reagents

1. Sterile Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)
2. Cobra venom factor (positive control) (Quidel Corp., A600)
3. Veronal buffer (Boston BioProducts, IBB-260)
4. MicroVue iC3b EIA kit (Quidel Corp., A006)
5. MicroVue C4d fragment EIA kit (Quidel Corp., A0008)
6. MicroVue Bb Plus EIA kit (Quidel Corp., A027)
7. Complement activator (Quidel, https://www.quidel.com/research/complement-reagents/complement-activator)
8. Pooled human plasma, anti-coagulated with sodium citrate
9. Cremophor, (Sigma, C 5135)
10. Doxil (Doxorubicin HCl, liposome, injection) This is a prescription medication available from licensed pharmacies; this drug may not be available to some research laboratories.
11. Taxol (Paclitaxel in Cremophor EL) This is a prescription medication available from licensed pharmacies; this drug may not be available to some research laboratories.

3.2 Materials

1. Pipettes, 0.05 to 1 mL
2. Microcentrifuge tubes, 1.5 mL
3. Pipet tips, 0.5 µL – 1.0 mL
4. Multichannel (8-12 channel) pipettor with 50-300 µL volumes
5. Conical tubes, 15 and 50 mL
6. Reagent reservoirs

3.3 Equipment

1. Microcentrifuge

2. Centrifuge, 2500x*g*, with a swinging basket for 5cc vacutainer tubes
3. Refrigerator, 2-8˚C
4. Freezer, -20˚C
5. Vortex
6. Incubator, 37˚C
7. ELISA plate reader capable of operating at 405 nm

4. Reagent and Control Preparation

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

4.1.1 Cobra Venom Factor (CVF)

Cobra Venom Factor (CVF) activates complement through alternative pathway. It is supplied as a frozen solution. Thaw according to the manufacturer's instructions and prepare daily use aliquots. This experiment requires 30 µL (1.1-50 U) of CVF solution. Store aliquots at a nominal temperature of -80˚C until assay performance is no longer acceptable. Avoid repeated freeze/thaw cycles. Discard any remaining sample from daily use aliquots upon completion of the assay.

4.1.2 Heat Aggregated Gamma Globulin (HAGG)

HAGG acts similar to naturally occurring immune complexes and is a very potent activator of complement through the classical pathway. This control is available from Quidel under the name "Complement Activator" (https://www.quidel.com/research/complement-reagents/complement-activator). Handle and store according to the manufacturer's instructions. Avoid repeated freeze/thaw cycles.

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 known to induce complement activation related pseudoallergy (CARPA) syndrome [2], and therefore is used as a nanoparticle relevant control. The following procedure can be used to prepare Cremophor-EL of similar composition to that

found in the clinical formulation of Paclitaxel (Taxol) (527 mg of purified Cremophor® EL (polyoxyethylated castor oil), 49.7% (v/v) dehydrated alcohol, 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 citric acid to mimic the concentrations in Taxol® and the generic formulation of paclitaxel.

4.2.2 Cremophor-EL formulated Paclitaxel (Taxol)

Taxol can be used 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 clinical pharmacies as 20 mg of Doxorubicin HCl in 10 mL vehicle. Store 2-8ºC.

4.3 Inhibition/Enhancement Control (IEC)

The IEC uses the positive control sample after incubation. Prior to loading this sample onto ELISA plate, add nanoparticles to the same final concentrations as in the study samples. For example mix 20 µL of the positive control sample and 10 µL of the test nanoparticle. The test result for this sample should be adjusted by the dilution factor 1.5 prior to comparison of this test value to the test value of the positive control sample. If the test results are ≤ 25% different, the test nanoparticle, at the given concentration, does not interfere with detection of complement split product by ELISA.

4.4 Negative Control (PBS)

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

4.5 Vehicle Control (specific to the given nanoparticle)

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

Dilute this sample the same way you dilute the test nanomaterials. This control can be skipped if nanoparticles are stored in PBS or saline.

4.6 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 nanoparticle solution dissolved/resuspended in PBS, at a concentration 3X the highest final test concentration. Test concentrations are based on the calculated 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 have been reviewed elsewhere [4] and are summarized in Box 1 below.

This assay evaluates four concentrations: 10X (5X if 10X cannot be achieved, or 100X or 30X when feasible) of the theoretical plasma concentration, the theoretical plasma concentration, and two serial 1:5 dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, 1 mg/mL is used as the highest concentration. Alternatively, the highest reasonably achieved concentration can be used if 1 mg/mL is unattainable.

Box 1. Example Calculation to Determine Nanoparticle Theoretical Plasma Concentration

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

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

The blood volume of a human is 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 a human. The theoretical plasma concentration, i.e. in vitro test concentration, is calculated by:

$$\text{theoretical plasma concentration} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg x } 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

For example, if the theoretical plasma concentration to be tested is 0.2 mg/mL, a stock of 6 mg/mL is prepared. This sample is then 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 sample dilutions 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 this assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL.

6. Plasma Collection and Storage

Blood is drawn into vacutainer tubes containing anticoagulant. Na-citrate is the ideal anti-coagulant for this assay. However, depending on phlebotomy paraphernalia, plasma anti-coagulated with Na-citrate may show high background in the ELISA assay. In this case, K_2-EDTA is an acceptable anticoagulant. The first 5-10 mL of blood should not be used to prepare plasma and should be discarded. For optimal results, it is important to keep blood at 20-24ºC, to avoid exposure to high temperatures (summer time) and low temperatures (winter time), and to avoid prolonged (>1 hr) storage. Blood should be transported to the lab in a contained Styrofoam box with warm packs (20-24°C).

To prepare plasma, blood is spun in a centrifuge for 10 minutes at 2500x*g*. Plasma is evaluated for the presence of hemolysis. Discolored plasma (an indication of hemolysis) should not be used to prepare the pool. Individual plasma specimens that showed no indication of hemolysis are pooled and mixed in a conical tube. Plasma must be used for complement testing within 1 hr after collection. Pooled plasma can be prepared by mixing plasma obtained from at least two donors. If analysis of individual donor plasma is needed, analyze at least three donors.

It is possible to use pooled sodium citrate plasma from commercial suppliers. However, be sure to notify the supplier the plasma is intended for complement testing to ensure there are no delays between blood draw and plasma collection. The supplier should freeze the plasma immediately after collection and ship to the lab on dry ice. When using frozen plasma for the complement activation assay, it is important to avoid repeated freeze/thaw cycles. 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 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

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.
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.
3. Prepare 100 μL aliquots and either use in EIA immediately or freeze at -20ºC for later analysis.
4. Follow the manufacturer's instruction to reconstitute complement standard, buffers and controls.
5. Dilute plasma samples prepared in step 3 in complement specimen diluent reagent (provided with each kit). Use the following guide for dilutions. Note however, dilution factors should be determined by each laboratory and adjusted if needed.
   iC3b: 1:1500 for positive control sample; 1:75 for negative control and other test samples
   C4d: 1:30 for all samples
   Bb: 1:75 for all samples
6. Follow manufacturer's instruction for plate loading volumes, incubation time and plate washing.

8. Data Analysis

Do not forget to use the appropriate dilution factor for control and study samples. Compare amount of complement components between positive control or study samples to that in the negative control. An increase in the complement component species ≥2.0-fold 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 to 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 the 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 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, may be less likely to cause hypersensitivity reactions.

9. Acceptance Criteria

1. Percent CV between replicates of standard curve, quality controls, and test samples should be within 25%.
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.
3. Run is acceptable if conditions described in 9.1 and 9.2 are met.
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.

10. References

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11. Abbreviations

CVF	cobra venom factor
PBS	phosphate buffered saline
EIA	enzyme immunoassay
HRP	horseradish peroxidase
IgG (H + L)	immunoglobulin G (high and low chains)
NC	negative control
PC	positive control