NCL Method ITA-5.2

Quantitative Analysis of Complement Activation

Nanotechnology Characterization Laboratory
National Cancer Institute-Frederick
SAIC-Frederick
Frederick, MD 21702
(301) 846-6939
ncl@mail.nih.gov

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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.

Method written by:

Marina A. Dobrovolskaia, Ph.D. (NCL)

Barry W. Neun, B.S. (NCL)

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 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)**.

This protocol is intended for follow-up studies on samples which demonstrated a positive response in the qualitative assay (NCL method ITA 5.1).

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 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.

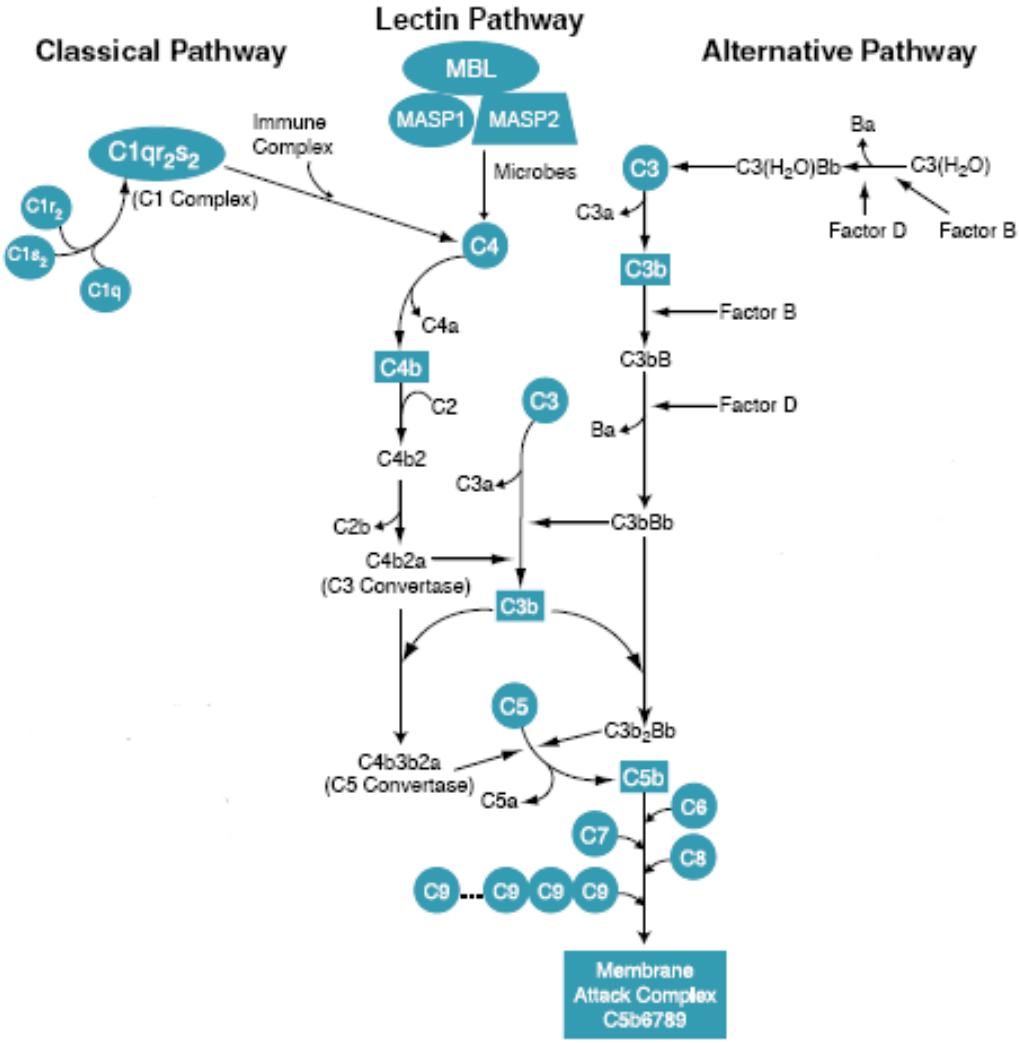

Figure 1. Complement activation pathways. (This illustration is reproduced from reference 1 with permission from EMD Biosciences, Inc.)

3. Reagents, Materials, and Equipment

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

3.1 Reagents

3.1.1 Sterile Ca^{2+}/Mg^{2+}-free Dulbecco's phospahte buffered saline (DPBS) (negative control) (Sigma, D8537)

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

3.1.3 Veronal Buffer (Boston BioProducts, IBB-260)

3.1.4 10% Tris-Glycine gels (Invitrogen, EC6075)

3.1.5 Pooled human plasma, anti-coagulated with Na-citrate

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

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

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

3.1.9 Doxil (Doxorubicin HCl, liposome, injection) (Manufactured by Ben Vanue Laboratories Inc. Distributed by Ortho Biotech Products LP; subject to prescription availability through the pharmacy.)

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

3.3 Equipment

3.3.1 Microcentrifuge

3.3.2. Centrifuge capable of running at 2500 x g, with a swinging basket set up to hold 5cc 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 capable of operating at 405 nm

4. Reagent and Control Preparation

4.1 Positive Control (Cobra Venom Factor)

Cobra Venom Factor is supplied as a lyophilized powder. Reconstitute the powder with water using volumes recommended by manufacturer. Prepare daily use aliquots and store at a nominal temperature of -80˚C as long as performance is acceptable. For this experiment, use 300 µL (1.1-50 U) of CVF solution. Avoid more than 2 freeze/thaw cycles.

4.2 Negative Control (DPBS)

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

4.3. 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.

4.4 Doxil

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.

5. Preparation of Study Samples

This assay requires 300 µL of nanoparticles. The following questions have to be considered when selecting the concentration: i) solubility of nanoparticles in a biocompatible buffer; ii) pH within physiological range; iii) availability of nanomaterial; and iv) stability in plasma. Whenever possible, the initial test should utilize nanoparticles with a stock concentration of 3.0 mg/mL, allowing for a final particle concentration of 1.0 mg/mL. When this concentration is not feasible, lower concentrations can be tested. In cases where physiologically relevant concentrations are higher than 1.0 mg/mL, nanoparticles should be tested at higher concentrations. Concentration is determined as total formulation, specific element (e.g. iron, gold, silver), or the active pharmaceutical ingredient (API). This is considered on a case-by-case basis depending upon the type of nanoparticle and its intended use. When buffer other then PBS

is used to reconstitute test-nanomaterial, a test sample composed of this buffer only should also be included in the analysis.

6. Plasma collection and storage

Blood is drawn into vacutainer tubes containing sodium citrate as an anticoagulant. The first 2-5 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 (summer time) and low temperatures (winter time), and to avoid prolonged (> 1 h) 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 2500 x g. 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.

It is possible to use pooled sodium citrate stabilized 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

7.1 In a microcentrifuge tube, combine equal volumes (300 μL of each) of veronal buffer, human plasma, and a test-sample (i.e., positive control, negative control, nanoparticles, or buffer used to reconstitute nanoparticles if different then DPBS).

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

7.4. Follow the manufacturer's instruction to reconstitute complement standard, buffers and controls.

7.5 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; 1:150 for negative control and nanoparticle-treated samples

C4d – 1:30 for all samples

Bb – 1:75 for all samples

7.6 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 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. Doxil is used in clinic and is known to induce complement activation related hypersensitivity reactions in sensitive patients **(3)**. 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.

9. Acceptance Criteria

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

9.2. 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.

9.3 Run is acceptable if conditions described in 9.1 and 9.2 are met.

9.4 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. Doxil is positive in the C4d EIA.

10. References

1. The Complement System. Complement reagents of the highest quality. EMD Biosciences, Calbiochem, Page 2. http://www.emdbiosciences.com/docs/docs/LIT/Complement_CB0617_EUSD.pdf
2. Xu Y., Ma M., Ippolito GC., Schroeder HW., Carrol MC., Volanakis JE. Complement activation in factor D-deficient mice. PNAS, 2001, 98, 14577-14582.
3. Chanan-Khan A, Szebeni J, Savay S, Liebes L, Rafique NM, Alving CR, Muggia FM. Complement activation following first exposure to pegylated liposomal doxorubicin (Doxil): possible role in hypersensitivity reactions. Ann Oncol. 2003, 14, 1430-1437.

11. Abbreviations

C	Celsius
CVF	cobra venom factor
DPBS	Dulbecco's phosphate buffered saline
EIA	enzyme immunoassay
g	gram
h	hour
HRP	horseradish peroxidase
IgG (H + L)	immunoglobulin G (high and low chains)
kDa	kilodaltons
mA	milliamps
min	minutes
mL	milliliters
MW	molecular weight
µL	microliters
NC	negative control
PC	positive control
PVDF	polyvinylidene fluoride
TBST	tris-buffered saline with Tween
U	units
V	volts