NCL Method ITA-5.1

Qualitative Analysis of Total Complement Activation by Western Blot

Nanotechnology Characterization Laboratory
Frederick National Laboratory for Cancer Research
Leidos Biomedical Research, Inc.
Frederick, MD 21702
(301) 846-6939
ncl@mail.nih.gov
http://www.ncl.cancer.gov

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:

Barry Neun, B.S.

Anna N. Ilinskaya, Ph.D.

Marina A. Dobrovolskaia, Ph.D

1. Introduction

This document describes a protocol for qualitative determination of total complement activation by Western blot analysis. 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 polyacrylamide gel electrophoresis (PAGE) and Western blot with anti-C3 specific antibodies. These antibodies recognize both the native C3 component of the complement and its cleaved products. When the test compound or positive control (cobra venom factor) induces activation of the complement system, the majority of the C3 component is cleaved and appearance of C3 cleavage products is documented.

This “yes” or “no” protocol is designed for rapid and inexpensive assessment of complement activation. Test nanoparticles found to be positive in this assay can subsequently undergo a more detailed investigation aimed at delineation of the specific complement activation pathway.

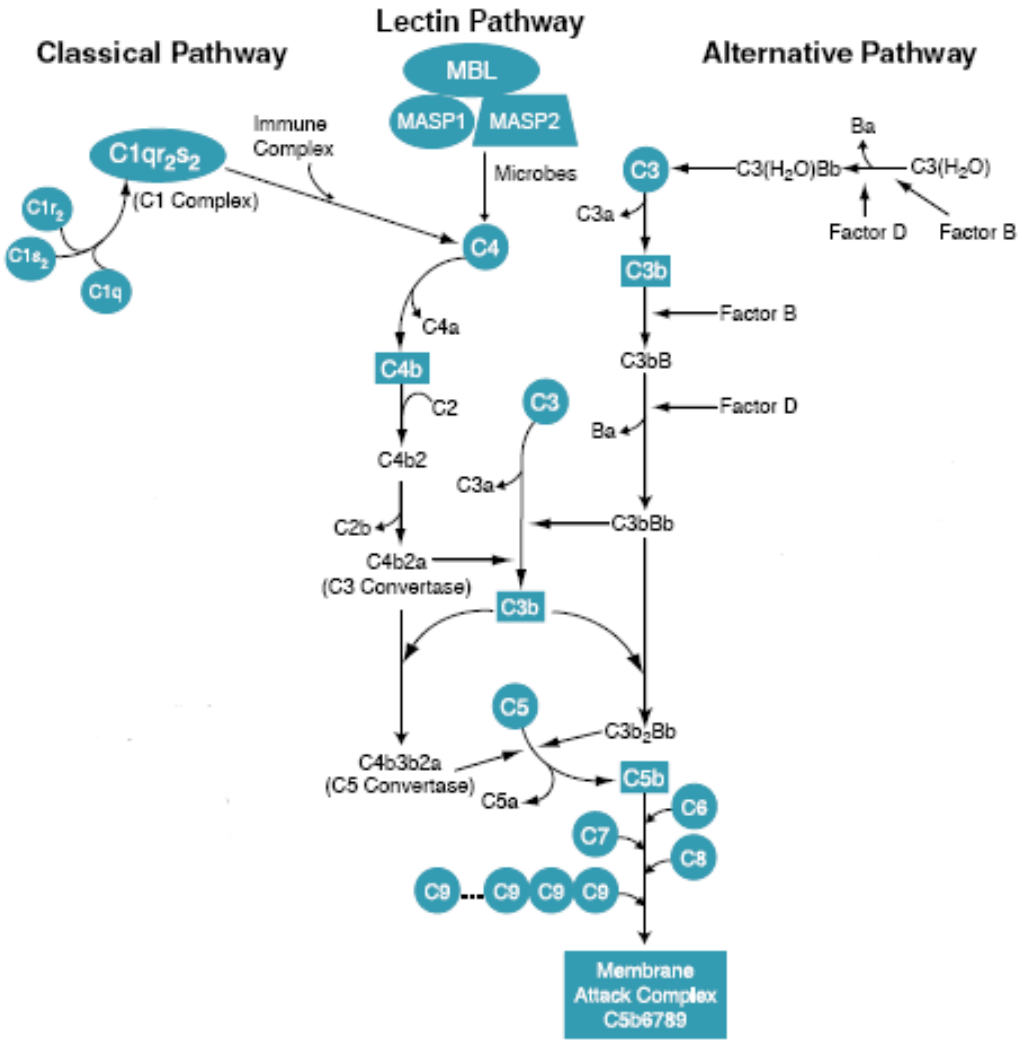

Figure 1. Complement activation pathways. (This illustration is reproduced from reference 1 with permission from EMD Biosciences, Inc.)

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 phospahte 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. 10% Tris-glycine gels (Invitrogen, EC6075)
5. Tris-glycine running buffer (10x) (Invitrogen, LC2675)
6. NuPAGE LDS 4x sample buffer (Invitrogen, NP0007)
7. Reducing agent (10x) (Invitrogen, NP0004)
8. Transfer Buffer (25x) (Invitrogen, LC3675)
9. Methanol (Sigma-Aldrich, 179337)
10. Tris-buffered saline (25x) (Amresco, J640-4L)
11. Tween 20 (Sigma, P7949)
12. Non-fat dry milk
13. Pooled human plasma, anti-coagulated with Na-citrate or K_2-EDTA
14. Westran S, PVDF protein blotting membrane (Schleicher & Schuell, 10 413 052)
15. Blotting paper (Schleicher & Schuell, CB-03)
16. Goat polyclonal anti-C3 antibody (EMD Biosciences Inc, Calbiochem, 204869)
17. Donkey anti-goat IgG(H+L) conjugated to HRP (Jackson ImmunoResearch Labs, 705-035-147)
18. ECL Western Blotting Substrate (Pierce, 32106)
19. Ponceau S (Fluka, 09276)
20. Hyperfilm ECL (Amersham Biosciences, RPN 2103K)
21. SeeBlue® Plus2 Pre-Stained Standard, (Invitrogen, LC5925)
22. Doxil (Doxorubicin HCl, liposome, injection) This is a prescription medication available from licensed pharmacies; this drug may not be available to some research laboratories.

3.2 Materials

1. Pipettes covering the range from 0.05 to 1 mL
2. Microcentrifuge tubes, 1.5 mL
3. Pipet tips, 0.5 µL – 1.0 mL
4. Gel-Loading tips
5. Hybridization bags
6. Saran Wrap
7. Scissors
8. Ruler
9. Film Cassette

3.3 Equipment

1. Microcentrifuge
2. Refrigerator, 2-8˚C
3. Freezer, -20˚C
4. Vortex
5. Incubator, 37˚C
6. Mini-gel protein electrophoresis system
7. Mini-gel blotting system
8. Rocking platform

4. Reagent and Control Preparation

4.1 Tris-Glycine Running Buffer

Prepare working solution by diluting 10X concentrated stock with distilled water. For example, mix 100 mL of stock with 900 mL of water. Use fresh.

4.2 Tris-Glycine Transfer Buffer with 20% methanol

Prepare working buffer from 25X stock solution by diluting 40 mL of stock in 760 mL of distilled water, then add 200 mL of methanol. Mix well. Chill before use. Use fresh.

4.3 TBST (TBS + 0.01% Tween 20)

Dilute 25X stock in distilled water by mixing 40 mL of the stock with 960 mL of water. Then add 100 µL of Tween20 and mix well. Unused buffer can be stored at room temperature overnight or up to 1 week at a nominal temperature of +4˚C.

4.4 Blocking Buffer (5% milk in TBST)

Dissolve 5 g of non-fat dry milk in 100 mL of TBST. Use fresh. Other blocking buffers may be used if they provide comparable sensitivity and performance.

4.5 Ponceau S Solution

Dilute stock solution with distilled water by mixing 10 mL of the stock solution with 40 mL of water. Mix well. Store at room temperature for up to 2 weeks.

4.6 Primary Antibody Solution

Thaw an aliquot of anti-C3 antibody and dilute 1:2000 in the blocking buffer. Use fresh.

Note: If antibody from a source other than that tested in validation is used, the final dilution of this antibody may have to be adjusted to provide a more optimal assay performance (i.e., minimum background, high signal-to-noise ratio).

4.7 Secondary Antibody Solution

Dilute donkey anti-goat IgG(H+L) HRP conjugate 1:50,000 in blocking buffer. Use fresh. Discard after use.

Note: If antibody from a source other than that tested in validation is used, the final dilution of this antibody may have to be adjusted to provide a more optimal assay performance (i.e., minimum background, high signal-to-noise ratio).

4.8 Positive Control 1 (Cobra Venom Factor)

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 10 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.

Note: If CVF is not available, other substances known to induce strong complement activation can be used, for example 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).

4.9 Positive Control 2 (nanoparticle relevant control)

PEGylated liposomal doxorubicin (Doxil) can be used as a nanoparticle relevant positive control. Doxil is supplied as a stock with a doxorubicin (DXR) concentration of 2mg/mL. When used in this assay, the final concentration of the sample is 0.67 mg/mL of DXR. Other positive controls can be used. However, some positive controls that may work well in ELISA (NCL ITA 5.2) may not be applicable to western blot. For example Cremophor-EL and Taxol alter protein mobility in the gel and should be avoided in this protocol.

4.10 Negative Control (PBS)

Sterile Ca2+/Mg2+ free PBS is used as the negative control. Store at room temperature for up to 6 months.

4.11 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.

5. Preparation of Study Samples

This assay requires 40 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 [3] 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.

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 0.01 mL of each of these sample dilutions is added to the test tube and mixed with 0.01 mL of plasma and 0.01 mL of veronal buffer, the final nanoparticle concentrations tested in this assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL.

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:

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

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 (10 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 at a nominal temperature of 37˚C for 30 minutes.
3. To each tube add 10 μL of 4X NuPAGE buffer supplemented with reducing agent, vortex, and heat at a nominal temperature of 95˚C for 5 minutes. Spin in a microcentrifuge at maximum speed for 30 seconds. Carefully transfer supernatants to clean tubes. At this stage, samples can be either used for further analysis, or frozen at a nominal temperature of -20˚C. If frozen, samples should be thawed at room temperature, vortexed, and briefly spun down before analysis.
4. Assemble gel running system. Prime wells with running buffer, then load protein marker and 3 μL of test samples and controls.
5. Run gel at 125 V for approximately 2 hr, or until dye reaches the bottom of the gel.

6. Rinse the gel with deionized water, wet the membrane with methanol and rinse with water. Assemble the protein transfer sandwich.
7. Perform protein transfer either overnight at 25-30 mA or 1-2 hr at 100 mA.
   Note: The conditions described in steps 5-7 above are optimal for mini-gels and Invitrogen protein electrophoresis and blotting systems. If other systems are used, other conditions may be applicable. Please refer to your equipment manufacturer's instructions.
8. Rinse membrane with deionized water.
9. Add 40 mL of Ponceau S solution and incubate on a rocking platform for approximately 5 minutes.
10. Wash the membrane with deionized water twice for approximately 10 min to remove excess Ponceau S stain. If staining reveals no problem with protein transfer such as air bubbles, smears, or unequal protein load in lanes, proceed to next step.
    Note: Steps 9-10 are optional. They are ideal to verify the quality of the transfer. If the laboratory has established a reproducibly good quality of transfer, these steps can be skipped.
11. Wash membrane with 50 mL of TBST for approximately 15 minutes on rocking platform.
12. Block the membrane with blocking buffer at room temperature for approximately 1 hr on rocking platform.
13. Incubate membrane with primary antibody solution for 90 min at room temperature on rocking platform.
14. Wash the membrane with 50 mL of TBST for 15-20 minutes on a rocking platform. Repeat for a total of two washes.
15. Incubate the membrane with the secondary antibody solution for 90 min at room temperature on a rocking platform.
16. Wash the membrane with 50 mL of TBST for 15-20 minutes on a rocking platform. Repeat for a total of two washes.
17. **Incubate membrane with ECL peroxidase substrate for approximately one minute and proceed with blot development immediately. If film is used, the exposure time is approximately 2-5 minutes. When imaging system is used, the optimal exposure time should be selected empirically for a given system.**

8. Data Analysis

The results are evaluated by densitometry using image analysis software such as NIH Image J [4].

9. Acceptance Criteria

1. Run is acceptable if both replicates of the positive and negative controls demonstrate acceptable performance, i.e. evident cleavage of C3 component of complement in former, and no or minor amount of cleaved C3 in latter (see Figure 2).
2. Positive response is estimated by the fold difference in the density of the split product in the sample as compared to that in the negative control. A ≥2.0-fold difference constitutes a positive response.
3. If one of the replicates of the positive or negative control fails to meet acceptance criteria in steps 1 or 2, entire run should be repeated.
4. If both replicates of a study sample demonstrate evident cleavage of the C3 component of the complement system, or one replicate is positive and the other replicate is negative, the sample is considered positive and should be analyzed further using a more thorough quantitative assay.
5. If neither replicate of a study sample demonstrates cleavage of the C3 component of the complement system, the sample is considered negative and no further analysis is required.

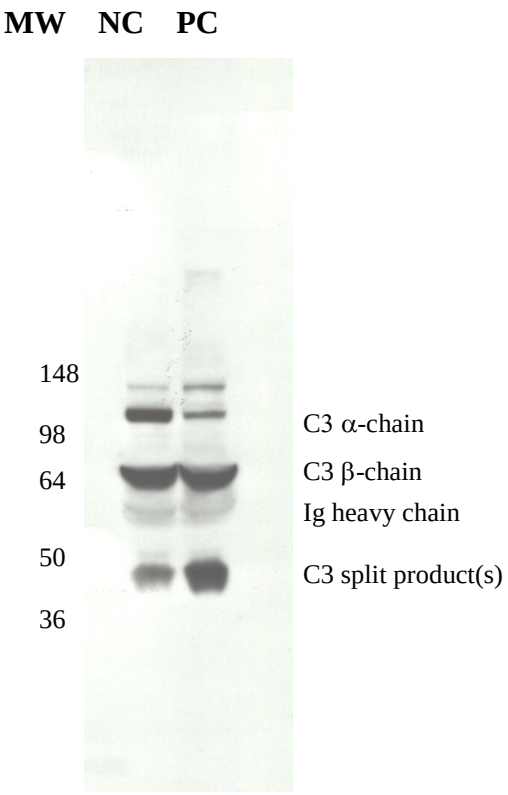

Figure 2. Western blot analysis of complement system activation. C3 (α chain) size is ~115 kDa; C3-cleavage product(s) (C3c, iC3b[C3α'] are ~ 43 kDa. NC is negative control (PBS); PC is positive control (cobra venom factor); MW is molecular weight protein marker. Note: The position of the MW denotes the position of the protein marker bands (not shown), not the molecular of the complement proteins depicted in the Western blot.

10. References

1. The Complement System. Complement reagents of the highest quality. Calbiochem, Page 2. http://www.ctr.com.mx/publicaciones/001CB0617.pdf
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3. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Control Release. 2013;172(2):456-66.
4. ImageJ software and plugins: http://rsb.info.nih.gov/ij/

11. Abbreviations

CVF	cobra venom factor
PBS	phosphate buffered saline
HRP	horseradish peroxidase
IgG (H + L)	immunoglobulin G (high and low chains)
kDa	kilodaltons
mA	milliamps
NC	negative control
PC	positive control
PVDF	polyvinylidene fluoride
TBST	tris-buffered saline with Tween