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
https://ncl.cancer.gov

https://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 W. Neun

Edward Cedrone

Marina A. Dobrovolskaia*

Nanotechnology Characterization Lab, Cancer Research Technology Program, Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute, Frederick, MD 21702

*- address correspondence to: marina@mail.nih.gov

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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 plasma protein mannose-binding lectin.

The complement system is a family of ~30 protein that include 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].

2. Principles

In the protocol presented herein, 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 will be a subject to a more detailed investigation aimed at delineation of the specific complement activation pathway.

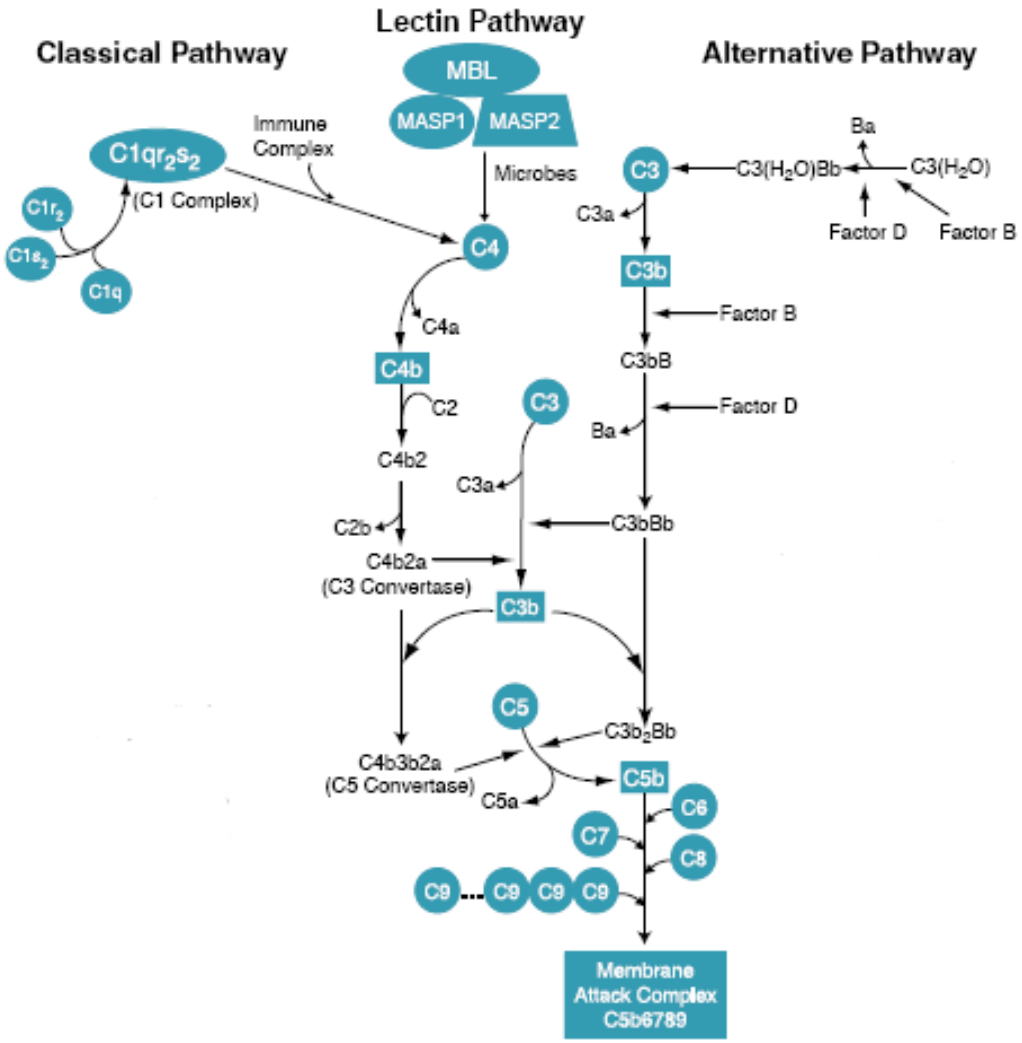

Figure 1. Complement activation pathways. (This illustration is reproduced from reference 1 with permission from EMD Biosciences, Inc.)

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; 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 10% Tris-Glycine gels (Invitrogen, EC6075)
- 3.1.5 Tris-Glycine Running Buffer (10x) (Invitrogen, LC2675)
- 3.1.6 NuPAGE LDS 4x sample buffer (Invitrogen, NP0007)
- 3.1.7 Reducing agent (10x) (Invitrogen, NP0004)
- 3.1.8 Pooled human plasma, anti-coagulated with Na-citrate or K2 EDTA
- 3.1.9 PVDF protein blotting membrane (Invitrogen, LC2002)
- 3.1.10 Blotting paper (Invitrogen, LC2002)
- 3.1.11 Transfer Buffer (25x) (Invitrogen, LC3675)
- 3.1.12 Methanol (Sigma-Aldrich, 179337)
- 3.1.13 Tris-Buffered Saline (25x) (Amresco, J640-4L)
- 3.1.14 Tween 20 (Sigma, P7949)
- 3.1.15 Non-fat dry milk
- 3.1.16 Goat polyclonal anti-C3 antibody (EMD Millipore, Calbiochem, 204869)
- 3.1.17 Donkey anti-goat IgG(H+L) conjugated to HRP (Jackson ImmunoResearch Labs, 705-035-147)
- 3.1.18 ECL Western Blotting Substrate (Pierce, 32106)
- 3.1.19 Ponceau S (Fluka, 09276)
- 3.1.20 Hyperfilm ECL (Amersham Biosciences, RPN 2103K)
- 3.1.21 SeeBlue® Plus2 Pre-Stained Standard, (Invitrogen, LC5925)

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

3.2 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 Gel-Loading tips

3.2.5 Hybridization bags

3.2.6 Saran Wrap

3.2.7 Scissors

3.2.8 Ruler

3.2.9 Film Cassette

3.3 Equipment

3.3.1 Microcentrifuge

3.3.2 Refrigerator, 2-8˚C

3.3.3 Freezer, -20˚C

3.3.4 Vortex

3.3.5 Incubator, 37˚C

3.3.6 Mini-gel protein electrophoresis system

3.3.7 Mini-gel blotting system

3.3.8 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 freshly prepared.

***Note**: If antibody from a source other than that tested in validation is used, the final dilution of this antibody can be adjusted to provide 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 freshly prepared. Discard after use.

***Note**: If antibody from a source other than that tested in validation is used, the final dilution of this antibody can be adjusted to provide 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) is supplied as a frozen solution. After initial thaw according to the manufacturer's instructions prepare daily use aliquots and store at a nominal temperature of -80˚C for as long as performance is acceptable. Avoid repeated freeze/thaw cycles, i.e. once a daily use aliquot is thawed, use it for the assay and discard any leftover amounts. For this experiment, use 10 µL (1.1-50 U) of CVF solution.

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 similarly to naturally occurring immune complexes and is 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) CVF activates complement through the alternative pathway.

4.9 Positive Control 2 (nanoparticle relevant control)

PEGylated liposomal doxorubicin (Doxil) can be used as 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 of them work well in ELISA (NCL ITA-5.2) but are not applicable to western blot. For example, Cremophor-EL and Taxol alter protein mobility in the gel and should be avoided in this protocol.

4.9 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.

4.10 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. Vehicle control should match formulation buffer of the test nanomaterial by both the composition and concentration. Dilute this sample the same way you dilute the test nanomaterials. If the vehicle is PBS this control can be skipped.

5. Preparation of Study Samples

This assay requires 40 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 [3] 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 0.01 mL of each of these samples 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 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. Na-citrate is ideal anti-coagulant for this assay, however depending on phlebotomy paraphernalia, plasma anti-coagulated with Na-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 prepared by mixing plasma obtained from at least 2 donors. If analysis of individual donor plasma is needed, analyze 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

7.1 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).
Note: Each sample is prepared in duplicate.

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 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 and carefully transfer supernatants to clean tubes.

Note: 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.

7.4 Assemble gel running system. Prime wells with running buffer, then load protein marker and 3 µL of test samples and controls.

7.5 Run gel at 125 V for approximately 2 hr, or until dye reaches a bottom of the gel.

7.6 Rinse the gel with deionized water, wet the membrane with methanol and rinse with water, then assemble protein transfer sandwich.

7.7 Perform protein transfer either overnight at 25-30 mA or 1-2 hr at 100 mA.

Note: Conditions described in sections 7.5-7.7 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.

7.8 Rinse membrane with deionized water.

7.9 Add 40 mL of Ponceau S solution and incubate on a rocking platform for approximately 5 minutes.

7.10 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 7.9 and 7.10 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.

7.11 Wash membrane with 50 mL of TBST for approximately 15 minutes on rocking platform.

7.12 Block the membrane with blocking buffer at room temperature for approximately 1 hour on rocking platform.

7.13 Incubate membrane with primary antibody solution for 90 min at room temperature on rocking platform.

7.14 Wash the membrane twice with 50 mL of TBST. Each wash step is 15-20 minutes and using rocking platform.

7.15 Incubate the membrane with the secondary antibody solution for 90 min at room temperature on a rocking platform.

7.16 Wash the membrane twice with 50 mL of TBST. Each wash step is 15-20 minutes and on rocking platform.

7.17 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

9.1 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 (e.g. see image on Figure 2).

9.2 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, or greater, difference constitutes a positive response.

9.3. If one of the replicates of the positive or negative control fails to meet acceptance criteria 9.1 and 9.2, entire run should be repeated.

9.4 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.

9.5 If both replicates of a study sample demonstrate no cleavage of the C3 component of the complement system, the sample is considered negative and no further analysis is required.

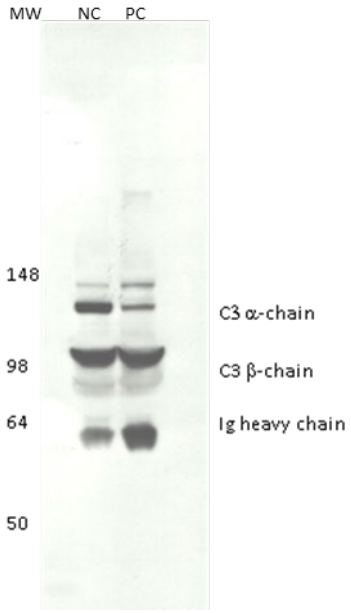

Figure 2. Analysis of complement system activation by Western blot. C3 (α chain) size is ~115 kDa; C3-cleavage product(s) (C3c, iC3b[C3α'] are ~ 43 kDa. NC is negative control (PBS); PC is positive control (cobra venom factor); MW is molecular weight protein marker.

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
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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.
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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