NANOTECHNOLOGY CHARACTERIZATION LABORATORY

NCL Method ITA-5
Version 1.0

Qualitative Analysis of Total Complement Activation by Western Blot

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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 is written by: _____ 5-22-06.

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Method validation was conducted on: 6/29/05 – 7/20/2005.

Date

Testing facility: NCI-Frederick. Bldg. 469, Room 250.

1. Introduction

This document describes a protocol for qualitative determination of total complement activation by Western blot. 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 (Figure 1). They are classical pathway, alternative pathway and lectin pathway. 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. Complement is a system composed of several components (C1, C2.....C9), and Factors (B, D, H, I, and P). Activation of either one of the three pathways mentioned above results in cleavage of C3 component of complement. In the protocol presented herein, human plasma is exposed to a test material and subsequently analyzed by polyacrylamide gel electrophoresis (PAGE) followed by Western blot with anti-C3 specific antibodies. These antibodies recognize both native C3 component of the complement and its cleaved products. Native C3 and no or minor amounts of C3 cleavage products are visualized by Western blot in control human plasma. When test compound or positive control (cobra venom factor), induce activation of complement, the majority of 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 for more detailed investigation aimed at delineation of specific complement activation pathway.

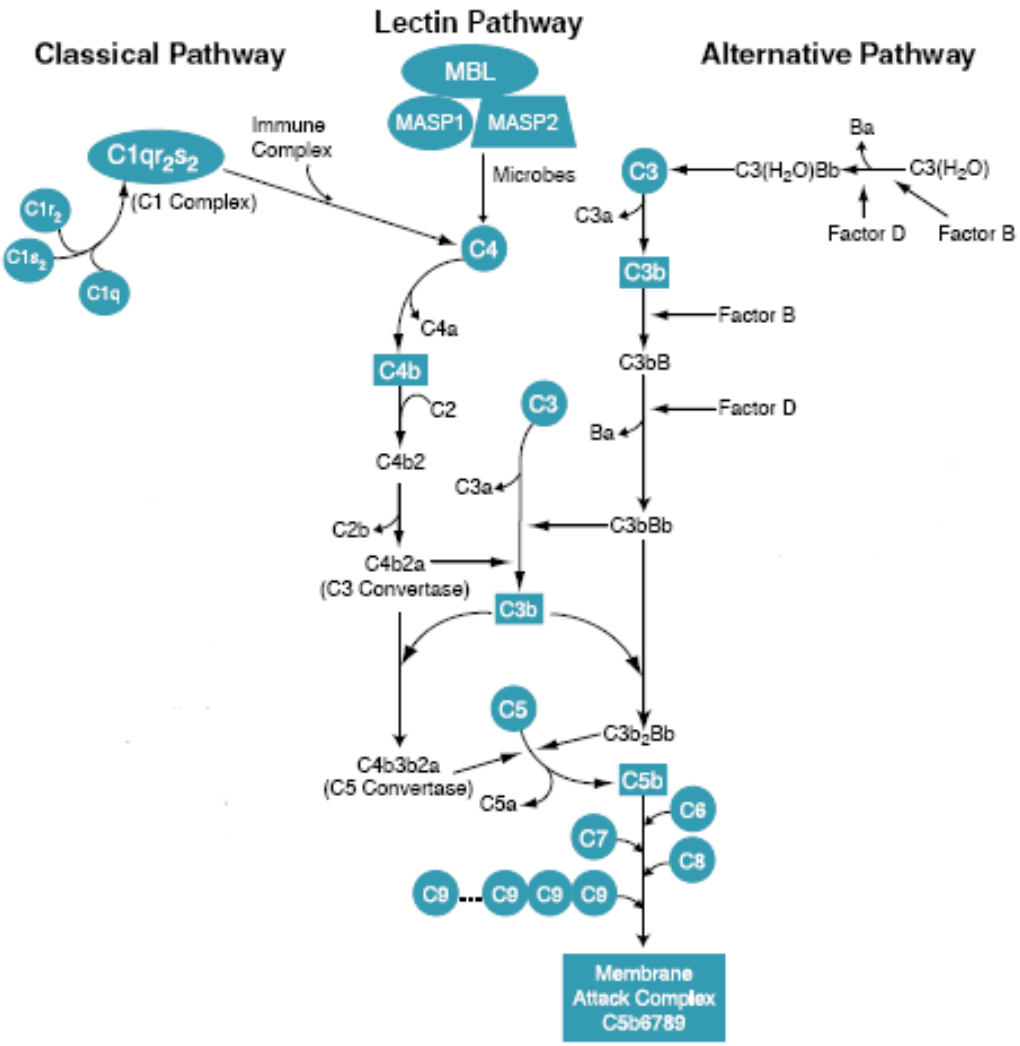


Figure 1. Complement activation pathways. (This illustration is reproduced from reference 1 with permission of EMD Biosciences Inc.)

2. Reagents

2.1. Sterile Ca^{2+}/Mg^{2+}-free DPBS, Sigma cat# D8537 (negative control)

2.2. Cobra Venom Factor, Quidel Corp., cat #A600 (positive control)

2.3. Veronal Buffer, Boston BioProducts, cat#IBB-260

2.4. 10% Tris-Glycine gels, Invitrogen, cat#EC6075

2.5. Tris-Glycine Running Buffer (10x), Invitrogen, cat#LC2675

2.6. NuPAGE LDS 4x sample buffer, Invitrogen, cat#NP0007

2.7. Reducing agent (10x), Invitrogen, cat#NP0004

2.8. Pooled human plasma anti-coagulated with Na-citrate

2.9. Westran S, PVDF protein blotting membrane, Schleicher&Schuell, cat#10 413 052

2.10. Blotting paper, Schleicher&Schuell, cat# CB-03

2.11. Transfer Buffer (25x), Invitrogen, cat#LC3675

2.12. Methanol, Sigma-Aldrich, cat#179337

2.13. Tris-Buffered Saline (25x), Amresco, cat# J640-4L

2.14. Tween 20, Sigma, cat# P7949

2.15. Non-fat dry milk

2.16. Goat polyclonal anti-C3 antibody, EMD Biosciences Inc, Calbiochem, cat#204869

2.17. Donkey anti-goat IgG(H+L) conjugated to HRP, Jackson ImmunoResearch Labs, cat#705-035-147

2.18. ECL Western Blotting Substrate, Pierce, cat#32106

2.19. Pontceu S, Fluka, cat#09276

2.20. Hyperfilm ECL, Amersham Biosciences, cat# RPN 2103K

2.21. SeeBlue® Plus2 Pre-Stained Standard, Invitrogen, cat#LC5925

Note: Equivalent reagents from other vendors can be used

3. Equipment

3.1. Pipettes covering range from 0.05 to 1 mL

3.2. Microcentrifuge tubes 1.5 mL

3.3. Microcentrifuge

3.4. Refrigerator, 2-8°C

3.5. Freezer, -20°C

3.6. Vortex

3.7. Pipet tips 0.5 µL – 1.0 mL

3.8. Gel-Loading tips

3.9. Incubator set at 37°C

3.10. Mini-gel protein electrophoresis system

3.11. Mini-gel blotting system

3.12. Hibridization bags

3.13. Saran Wrap

3.14. Scissors

3.15. Ruler

3.16. Rocking platform

3.17. Film Cassette

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 40mL of stock in 800mL 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 40mL of the stock with 960mL 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 5g of non-fat dry milk in 100 mL of TBST. Use fresh. Other blocking buffers may be used is 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 (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 the experiment use 10 μL (1.1-50 U) of CVF solution. Avoid more than 2 freeze/thaw cycles.

4.9. 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.

5. Preparation of Study Samples

This assay requires 20 μL of nanoparticles (10μL per sample to be analyzed in duplicate). 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. For the initial screen the test concentration is selected based on results from general toxicity assays. A nanomaterial, which revealed toxicity in general toxicity assays, is tested at two concentrations selected

at the low and the high end of the dose response curve. A nanomaterial, which did not reveal toxicity in a general toxicity assays is tested at one concentration equal to highest dose tested in general toxicity assay. When buffer other then PBS is used to reconstitute test-nanomaterial an additional sample composed of this buffer only should be included in analysis.

6. Experimental Procedure.

6.1. In an 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 buffer used to reconstitute nanoparticles if different then DPBS).
Note: each sample is prepared in duplicate.

6.2. 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 1 hour.

6.3. 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 a maximum speed for 30 minutes and carefully transfer supernatants to clean tubes.
Note: at this stage samples can be either used for further analysis of frozen at a nominal temperature of -20°C. If frozen, samples should be thawed at room temperature, vortexed and briefly spun down before analysis.

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

6.5. Run gel at 125V for approximately 2 hours or until dye reaches a bottom of the gel.

6.6. Rinse the gel with deionized water and assemble protein transfer sandwich.

6.7. Perform protein transfer either overnight at 25-30 mA or 1-2 hours at 100mA.
Note: Conditions described in sections 6.5-6.7 above are optimal for minigels 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.

6.8. Rinse membrane with deionized water.

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

6.10. Wash the membrane with deionized water twice for approximately 10 minutes to remove excess of Ponceau S stain. If staining reveals no problem with protein transfer such as air bubble, smears, not equal protein load in lanes, proceed to next step.

6.11. Wash membrane with 50 mL of TBST for approximately 15 minutes.

6.12. Block the membrane with blocking buffer at room temperature for approximately 1 hour.

6.13. Incubate membrane with primary antibody solution for 90 minutes at room temperature.

6.14. Wash the membrane twice with 50 mL of TBST. Each wash step is 15-20 minutes.

6.15. Incubate the membrane with the secondary antibody solution for 90 minutes at room temperature.

6.16. Wash the membrane twice with 50 mL of TBST. Each wash step is 15-20 minutes.

6.17. 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.

7. Acceptance Criteria

7.1. 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.

7.2. If one of the replicates of the positive or the negative control fails to meet acceptance criterion 7.1, entire run should be repeated.

7.3. If both replicates of a study sample demonstrate evident cleavage of the C3 component of complement, or one replicate is positive and the other replicate

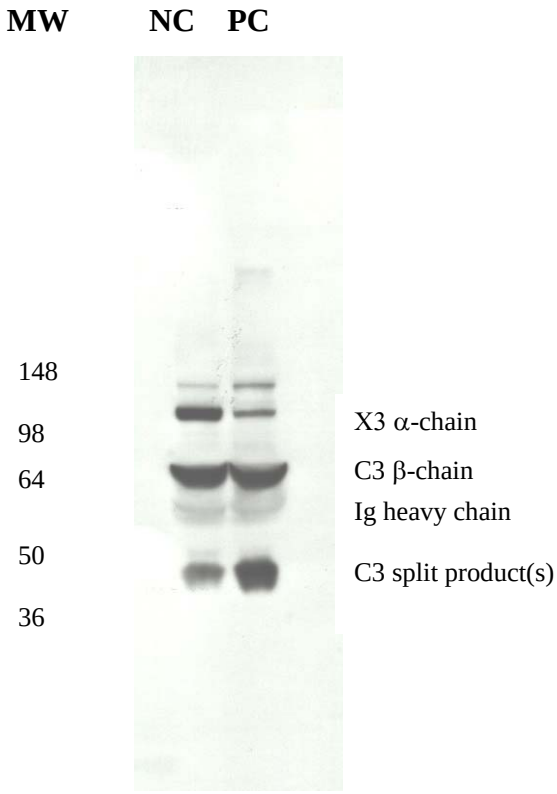
demonstrate intermediate cleavage, the sample is considered positive and should be analyzed further using more thorough quantitative assay.

7.4 If one replicate of a study sample demonstrates positive response and second replicate is negative, then this sample should be re-analyzed.

7.5. If both replicates of a study sample demonstrate no obvious cleavage of the C3 component of complement, the sample is considered negative and no further analysis in quantitative assay is required.

7.6. If both replicates of a study sample demonstrate intermediate cleavage of the C3 component of complement, the sample is considered positive and should be further analyzed using quantitative assay.

8. Example of Western Blot



On the image above, NC is negative control, PC is positive control and MW is molecular weight protein marker. C3 (α chain) size is ~115 kD, C3-cleavage product(s) (C3c, iC3b[C3α'] are ~ 43 kD.

9. References

9.1. 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

9.2. Xu Y., Ma M., Ippolito GC., Schroeder HW., Carrol MC., Volanakis JE. Complement activation in factor D-deficient mice. PNAS, 2001, 98, 14577-14582.