Chapter 16

C1q Assay for the Detection of Complement Fixing Antibody to HLA Antigens

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Abstract

Solid phase Luminex® and flow cytometric single antigen bead assays offer exquisite sensitivity and specificity for HLA antibody detection. Unlike the historical complement-dependent cytotoxicity (CDC) method, these assays do not distinguish complement fixing from non-complement fixing antibody, the former of which are considered the most clinically relevant in the peri-transplant period. This chapter describes a novel solid phase C1q binding assay to distinguish HLA antibodies that can bind the first component of complement (C1q). These antibodies have the capacity to initiate the complement cascade irrespective of whether that actually occurs. The C1q assay detects many more complement fixing antibodies than are observed by the less sensitive and less specific CDC assay.

Key words HLA antibody, Solid phase assay, Single antigen beads, Luminex®, Transplantation, Complement, C1q

1 Introduction

The complement-dependent cytotoxicity (CDC) method has been used for more than 40 years to define clinically relevant HLA antibodies that have a very high correlation with adverse outcome in the transplant setting when they are specific to the donor [1]. The test uses rabbit, not human, complement and is both insensitive and nonspecific. The advent of solid phase immunoassays on the Luminex® platform and flow cytometric bead assays have overcome the sensitivity and specificity drawbacks of the CDC method [2, 3]. These assays detect IgG antibody that can bind to HLA class I (A,B,C) and class II (DR,DQ,DP) antigens present on beads. However, they do not distinguish complement fixing from non-complement fixing antibody. A series of modifications to the solid phase assays have been developed [4–8] to define clinically important antibodies that can bind the complement component, C4d. The C4d assays are functional assays, requiring progression through the early part of the complement cascade, use a variable source of

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human complement and have low sensitivity with maximum mean fluorescence intensity (MFI) on the beads of ~3,500 [5].

The C1q assay [9], developed on Luminex® single antigen beads, measures the binding of C1q to the antibody and detects the array of antibodies capable of binding complement and initiating the classical pathway, irrespective of whether the cascade is actually activated. It does not require complement activation and is not affected by complement regulatory factors, except perhaps by C1INH [10]. The method has undergone incremental developmental modifications including two different two-step methods using autologous serum as the source of human C1q (hC1q) or spiking with purified hC1q and detecting bound C1q with purified custom labeled sheep anti-human hC1q (*see* **Notes 1** and **2**). The current C1q assay is a one-step assay (*see* **Note 3)** in which the beads are incubated with patient serum and a standard amount of purified, directly PE-labeled hC1q, simultaneously. The assay is highly sensitive, with maximum MFI values on the SAB of greater than 30,000. The assay also detects IgM. While this could be considered a potential drawback, the definition of IgM+ DSA has been shown to be clinically relevant [9]. Likely because of its ability to detect both IgG and IgM that is DSA, the C1q assay is always positive when the CDC is positive. Remarkably, it detects 513 % more antibodies capable of fixing complement than CDC when compared with the universe of IgG positive antibodies detected by IgG [9]. The C1q assay is highly correlated with clinical outcome in kidney and heart transplant recipients [11–13].

2 Materials

2.1 Reagents

1. LABScreen Class I Single Antigen Bead Mix (OneLambda LS1A04, Canoga Park, CA). Store at 4 °C.
2. LABScreen Class II Single Antigen Bead Mix (OneLambda LS2A01, Canoga Park, CA). Store at 4 °C.
3. 10× Wash Buffer (LABScreen, OneLambda, Canoga Park, CA). Store at 4 °C. If crystals are present, dissolve in a 37 °C water bath before use.
4. 1× Wash Buffer (sufficient for 48 wells): 5 mL 10× Wash buffer, 45 mL double-distilled water (Mix well).
5. Bio-C1q: Biotin-labeled human complement component C1q (One Lambda, Canoga Park, CA). Store at −70 °C.
6. SA-PE: Phycoerythrin-conjugated streptavidin (OneLambda LT-SAPE, Canoga Park, CA). Lyophilized. Store at 4 °C. DO NOT FREEZE.
7. HEPES Buffer: 10 mM HEPES, 0.3 M NaCl, pH 7.2. Store at room temperature.

8. Deionized H_2O.
9. 20 % bleach in deionized H_2O.
10. LiquiChip Calibration Bead Kit containing Cal1 and Cal2 beads (Qiagen, Valencia, CA).
11. LiquiChip Control Bead Kit containing Con1 and Con2 beads (Qiagen, Valencia, CA).
12. Absorption Beads: Polystyrene particles 0.4–0.6 μm diameter, in solution (Spherotech, Lake Forest, IL).

2.2 Equipment and Supplies

1. LABScan 100 or 200 flow analyzer (also known as LiquiChip or Luminex® instrument).
2. Luminex® XY platform.
3. Eppendorf 5810R/Sorvall Legend Centrifuge with holders for 96-well microplates.
4. Vortex Genie 2 mixer.
5. IKA MS3 digital microplate shaker.
6. Barnstead/LabLine horizontal tray shaker (Model 4625).
7. Eppendorf Centrifuge 5424.
8. Electronic pipettes and pipette tips.
9. Greiner Bio-One V-Bottom 96-well microplates (VWR 651201).
10. Reagent boats (Art Robbins Instruments, Sunnyvale, CA).
11. 0.2 mL PCR strip vials.
12. Bullet tubes: 0.6 mL microcentrifuge tubes.
13. Fusion analysis program (One Lambda, Canoga Park, CA).

3 Methods

3.1 Serum Preparation

Keep sera on ice or at 4°C at all times unless otherwise indicated.

1. (Samples must be frozen before testing). Thaw serum on ice.
2. Centrifuge serum at 21,130 rcf for 10 min in the Eppendorf 5424 centrifuge prior to test.
3. Transfer serum to a clean, labeled tube avoiding any sediment at the bottom.
4. In a bullet tube, mix Spherotech adsorption beads with serum at 1:10 ratio, i.e., one part beads plus ten parts serum, e.g., 3 μL of beads + 30 μL of patient serum per test (*see* **Note 4**).
5. Incubate 10 min at room temperature with gentle shaking on Vortex Genie 2 (speed 6).
6. Pellet beads by spinning for 10 min in an Eppendorf 5424 centrifuge at maximum speed.

7. Transfer the serum into a clean, labeled bullet tube containing 3 μL Spherotech beads.
8. Repeat **steps 5** and **6**.
9. Transfer the absorbed serum into a clean, labeled bullet tube.
10. Arrange tubes according to the plate plan.

3.2 Testing

1. Remove single antigen beads from refrigerator and verify bead lot numbers (*see* **Note 5**).
2. Vortex bead mixture vigorously.
3. Use a multi-dispense pipette to add 2.5 μL of appropriate beads to the bottom of each test well.
4. Use a Rainin 5–50 μL multichannel pipette to dispense 10 μL of each serum sample into the corresponding test well. *Be sure that tips are equally filled for each serum sample, and no air bubbles are present. Verify that all 10 μL have been dispensed into the test wells* (*see* **Note 6**).
5. Vortex the plate on the IKA shaker 30 sec at 750 RPM (*see* **Note 7**).
6. Add 10 μL Bio-C1q to each test well using the same Rainin multichannel pipette.
7. Vortex the plate on an IKA shaker 30 sec at 750 RPM.
8. Cover plate with aluminum foil to keep it dark.
9. Place the covered plate on the Barnstead/Lab line tray shaker.
10. Incubate the plate in the dark at room temperature, shaking at speed 2.5, for 30 min.
11. Make the SAPE dilution 2 min before end of the first incubation. SAPE is diluted according to lot specification of Bio-C1q and SAPE. Dilution factor varies with each lot. 10 μL of diluted SAPE is needed for each test well. Add the HEPES buffer first to the dilution tube. Add the correct amount of SAPE to the HEPES buffer with 1–10 μL Rainin pipette at speed 3. Rinse the pipette tip once (*see* **Note 8**).
12. When the incubation is finished, use the same Rainin pipette to add 10 μL diluted SAPE to each test well.
13. Vortex the plate on an IKA shaker for 30 sec at 750 RPM.
14. Cover plate with aluminum foil to keep it dark.
15. Place the covered plate on the Barnstead/Lab line tray shaker.
16. Incubate the plate in the dark at room temperature, shaking at speed 2.5 for 20 min.
17. During the incubation, make 1× wash buffer from 10× stock solution, as per instructions in Subheading 2.1 (**step 4**). 500 μL per test well is required.
18. At the end of the incubation, add 100 μL 1× wash buffer to each well.
19. Vortex the plate on an IKA shaker for 30 sec at 750 RPM.

20. Add another 100 μL of 1× wash buffer.
21. Cover and centrifuge the plate at 1,500 × *g* in Eppendorf 5810R centrifuge for 3 min.
22. Immediately flick the plate **hard** to remove the liquid and blot upside down twice by banging the tray on a stack of paper towels *BEFORE TURNING TRAY RIGHT SIDE UP* (*see* **Note 9**).
23. Resuspend the dry pellet on the IKA shaker at 1,300 RPM for 30 sec.
24. Repeat wash **steps 18–22** once more, for a total of two washes.
25. Add 60 μL 1 × wash buffer to each test well.
26. Vortex the plate on an IKA shaker for 30 sec at 750 RPM.
27. Acquire sample data using the LABScan 100 Flow Analyzer (Luminex® instrument) (*see* **Notes 10** and **11**).

3.3 Analysis and Interpretation

1. Open HLA Fusion software.
2. Log in.
3. Click *Analyze Data* and *Labscreen*.
4. Select the run from Z:\HLAVNet\LABSCREEN_OUTPUT\OUTPUT.
5. Check mark *ABNeg sample* as control.
6. Click on *Import*. The run will appear in the "Navigator" screen (*see* **Note 12**).
7. Select *Baseline* in the lower left corner of the screen.
8. Arrange the baseline MFI values from highest to lowest and reading from the lowest up, find the first increment of 300 MFI or greater. Record the MFI value of the lower MFI bead where that break occurs.
9. Click on *Graph Raw* in upper right corner of screen.
10. On the graph in Fusion, drag all cutoff lines to the MFI value that you have just recorded (i.e., all MFIs above this cutpoint are Positive and have a score of eight and all MFIs below this cutpoint are negative and have a score of one). Scores are automatically assigned by Fusion after the lines have been moved.
11. Add 1,000 MFI to the MFI value of the highest negative bead (i.e., highest MFI value with a score of one).
12. *Positive* calls start at this value or higher (sum of highest negative MFI plus 1,000).
13. Any reaction with a score of eight but below the Positive antibody threshold is called *Possible*.
14. Double-click specificities to add them to the "Final Assignment" box.
15. Click *Save*.
16. Report.

4 Notes

1. The C1q method was modified by adding a standard (physiologic) amount of exogenous purified hC1q to the patient serum prior to incubation with the beads when it became obvious that patients with protein losing enteropathy did not have sufficient native C1q for the assay to work.
2. The C1q method is equally applicable to any solid platform including the flow cytometer.
3. Because the procedure is a one-step procedure, dithiothreitol (DTT) cannot be used to reduce IgM antibody. The C1q molecule is a six part structure which is held together by disulfide bonds. Thus, reducing the disulfide bonds for IgM also destroys the C1q. Reduction of IgM from the serum by heat treatment (63 °C × 13 min) can remove high background but the effect on the patient-specific antibody is currently unknown. Removal of IgM is best achieved by adsorption with beads specific for IgM (i.e., coated with anti-IgM antibody) which are currently custom reagents.
4. Absorption is CRITICAL to achieving valid test results. It removes sporadic blocking factors of unknown origin which maximize the signal to noise ratio and results in valid controls. In the absence of absorption, the positive control (bead #2) can be very low (~300 MFI) making the test invalid. In some cases, absorption with the usual absorption beads is inadequate to remove high background on the negative control (bead #1). In rare instances, the negative control value can be >20,000 MFI. This can be due to IgM which must be removed to obtain a valid test.
5. The thaw date must be entered on the Bio-C1q bottle label. The expiration date is 3 months from the thaw date and must not exceed original kit expiration date.
6. Because the volumes of beads and serum are so small per test well, it is essential to assure that the serum is fully coating the beads and not dispersed in bubbles around the circumference of the well. This step is CRITICAL.
7. The vortexing is critical to the reproducibility of the assay. It is important to use a validated shaker at the programmed speed to have consistency between technologists and between runs.
8. SAPE is not stored after dilution. Make only as much as you need. The first time this step is performed, it is likely to take more than 2 min. It is important to have all details regarding the required dilution factor ready.
9. The flicking and blotting steps are CRITICAL to the success of the assay. The beads are well embedded in the V-bottom

wells. As soon as the centrifugation is over, it is important to flick the liquid off hard into the sink and bang the wells (upside down) on a pad of paper towels to get off ALL the liquid.

10. Collect a minimum of 100 of each bead to assure validity of results.
11. Currently the LabXpress robot (One Lambda, Canoga Park, CA) is not programmed for testing by C1q. It cannot be used for methods using less than 5 μL of beads.
12. Before the run is analyzed, check the negative and positive controls to make sure they fall within acceptable limits (predetermined by local validation). In general, the negative control (Bead #1 on the SAB) should be less than 500 MFI and the positive control (Bead #2 on the SAB) should be more than 3,000 MFI. The positive control bead was not designed for the C1q assay and is a bead coated with IgG. However, achieving values over 3,000 MFI should be routine and many go up to 12,000 MFI.

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