Chapter III: Flow Cytometry and Luminex

Section: Application Module

Module: DSA Assay: A Donor-Specific Solid-Phase Method for Detecting anti-MHC Antibodies using XMap™ (Luminex™) Technology

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**Background:**

The DSA (donor specific antibody) assay is a Luminex-based solid phase immunoassay designed to detect IgG antibodies binding to solubilized, donor-derived HLA Class I and HLA Class II proteins.

“Capture” beads covalently linked with monoclonal antibodies specific for either MHC I or MHC II allow the user to create a solid-phase surface (beads) for specifically binding donor MHC proteins. Once the beads are coated, non-bound proteins in the donor lysate are removed by thorough washing. These beads can now be used to probe patient sera for the presence or absence of donor-specific alloantibodies.

Because the beads capturing MHC Class I proteins are distinct from the beads capturing MHC Class II proteins (each type of bead has an intrinsic fluorescent emission which the Luminex can readily differentiate), MHC Class specificity is easy to determine in this assay system. This offers a significant advantage over targets such as donor B and T lymphocytes, routinely used in crossmatch assays.

Peripheral blood leukocytes and platelets, spleen, and/or lymph nodes can all be used as donor source material in this assay. Solubilized HLA proteins are obtained from donor source material following exposure of the isolated cells to a lysing detergent. Cell fragments are removed by centrifugation and the lysate can be used immediately or stored for future testing.

A series of control beads, which are also distinguished by their different fluorescent emissions by the Luminex instrument, are used to determine: 1) the amount of nonspecific IgG binding to the solid matrix beads, and 2) that the appropriate conjugate was employed during each assay. There are two different secondary conjugates included in the kit. The first conjugate (PE-anti-human IgG) binds to human IgG. The second conjugate (PE-streptavidin) is used to detect biotinylated monoclonal antibodies used to determine: 1) the amount of donor MHC Class I proteins bound to the Class I capture beads, and 2) the amount of donor MHC Class II proteins bound to the Class II capture beads.

**Specimens**

**Recipient sera**

1. A sterile clotted blood sample with no anticoagulant is preferred.
2. Re-calcified plasma can also be used.
3. Unacceptable serum specimens
   a. Avoid specimens at risk for reduced antibody activity such as those exposed to excessive heat or repeated freeze-thaw cycles
   b. Avoid specimens with bacterial or fungal contamination
   c. Avoid specimens containing visible aggregates and/or fibrin. Pre-clear these specimens by spinning at high rpm (e.g. 15,000 rpm in a serofuge)

Donor material
   1. Anti-coagulated (ACD or heparin) blood
   2. Donor spleen
   3. Donor lymph node

Reagents and Supplies

1. DSA kits are commercially available and include all necessary reagents to evaluate up to 92 determinations depending on the batching stratagem (single donor/multiple sera or multiple donors/single or multiple sera).

Contents of DSA kit:
   1. Lymphoocyte Lysis Buffer: used to prepare donor lysates
   2. Specimen Diluent: used to dilute patient, control sera, and Lysate Control Reagent
   3. DSA Capture beads: a mixture of 7 beads, one to capture donor MHC I, one to capture donor MHC II, 3 beads to monitor nonspecific “background” IgG binding, and 2 beads to insure appropriate conjugate use.
   4. Lysate Control Reagent (LMLCR): biotinylated anti-MHC I and MHC II monoclonal antibodies
   5. SA-PE: strepavidin-Phycoerythrin, a conjugate to bind to the biotinylated anti-MHC monoclonal antibodies
   6. Conjugate: PE-anti-human IgG
   7. Wash Buffer
   8. Positive Control Serum for the supplied Dried Lymphocyte Control
   9. Negative Control Serum for the supplied Dried Lymphocyte Control
   10. Dried Lymphocyte Control Pellet: to provide a lysate standard

Not included in kit:

1. 96-well microtiter filter plates for use on vacuum manifold.
2. Polypropylene tubes for lysate/bead preparation, dilutions of control sera, and dilutions of secondary antibodies.
Instrumentation/Special Equipment

1. LABScan™ Flow Analyzer (Luminex)
2. Vacuum manifold for plate washing
3. Single and/or multichannel pipetters
4. Tabletop microcentrifuge (e.g. a Fisher centrifuge) and rotor capable of reaching forces up to 2000xg.
5. Vortex Mixer

Preparation of Lysate

Reagents and Supplies

1. Donor source material
   a. Anti-coagulated (ACD or heparin) donor blood
   b. Donor lymph node
   c. Donor spleen
2. PBS
3. Ficoll-Hypaque
4. Culture Media
5. Lymphocyte Lysis Buffer (supplied in kit). Dilute 1:10 using deionized, double distilled water. A 50 ml solution (5ml concentrate+ 45 ml dd water) can be prepared and stored at 4°C.

Procedure

1. Isolate leukocytes using standard Ficoll-Hypaque separation.
2. Wash cells 3 times with culture media at 1200 rpm (200xg) for 10 minutes.
3. Disperse the cell pellet and resuspend in 5-10 ml culture media.
4. Transfer the cells into tubes whereby the packed cell volume can be easily estimated (microcentrifuge tubes or a 15 ml conical centrifuge tube).
5. Obtain a packed cell pellet and estimate the volume by comparing with a known volume of liquid placed in a similar tube.
6. Remove as much liquid from the pellet as possible.
7. Disperse the packed cells using a vortex mixer.
8. Add diluted lysis buffer using 10X the packed cell volume.
9. Mix with a pipette or vortex to completely lyse the cell pellet.
10. Transfer lysates to microcentrifuge tubes and spin at 200xg for 2 minutes.
    Packed cell fragments will be white and supernatant will be blue to green/purple depending on red cell contamination.
11. Combine lysates into one tube, mix well, and aliquot into labeled freezing vials.
12. Store at -70 to -80°C.
Procedure Notes

1. A procedure based on number of cells is also an option. This approach works well when starting material is a limited amount of peripheral blood. ~2.2X10^6 leukocytes will yield 8ul of lysate which is required for each assay well.

2. The amount of Class I and Class II expressing cells determines the quality and quantity of donor lysate. Since the intent is to maximize both Class I and Class II proteins, minimize washing to maximize cell recovery (e.g. platelets are a rich source of Class I and may be lost during excessive washing.)

3. Avoid cell clumping prior to exposure to lysing buffer, as this can result in poor lysates and diminished MFI values for the lysate control beads. For example, freeze-thawed cells may need to be treated with DNase to prevent clumping prior to addition of lysis buffer.

4. Red cell contamination should not interfere with the assay. However, an estimate of the packed cell volume with heavy red cell contamination may be impossible. Hypotonic shock can be used to easily remove red cells, especially when donor spleen is used as a starting material.

5. When working with relatively small volumes (less than 10ml) it is convenient to use Fisher tubes and Fisher centrifuges with a 12 tube rotor.

Once the lysate is prepared, there are 3 simple phases to performing the assay. Each requires 30 minutes. The phases are: 1) coating the capture beads with donor MHC Class I and Class II molecules, 2) incubation with patient’s serum, and 3) detecting bound IgG.

Preparation of reagents prior to assay

Before starting, bring Wash Buffer and Specimen Diluent to room temperature (20-25°C). For each donor lysate, determine the volumes of reagents that will be required for each donor/recipient assay by using the following tables. In the example shown in Table 1, the user will perform an assay using 4 wells (one additional well is included for losses due to pipetting). For each donor lysate used, one well will test for the amount of MHC Class I and Class II captured (the LMLCR control well), one well can be used for the laboratory’s negative serum control, one well can be used for the laboratory’s positive serum control, and one well can be used for a patient serum sample.

As an assay control, a lysate prepared from the Dried Lymphocyte Control is also tested; one well will be used to determine the amount of MHC Class I and Class II captured (the LMCR control well), one well will be used for the negative control serum supplied in the kit, and one well will be used for the positive control serum supplied in the kit. (See Quality Control section below for additional information. This control is not included in the Preparation tables shown below.)
Table I: Bead Preparation

<table>
<thead>
<tr>
<th></th>
<th># of wells</th>
<th>Volume per well</th>
<th>total volume</th>
<th>purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture beads</td>
<td>5</td>
<td>5 ul</td>
<td>25 ul</td>
<td>Coat beads</td>
</tr>
<tr>
<td>Lysate</td>
<td>5</td>
<td>8 ul</td>
<td>40 ul</td>
<td>Coat beads</td>
</tr>
<tr>
<td>Wash Buffer</td>
<td>5</td>
<td>42 ul</td>
<td>210 ul</td>
<td>Coat beads</td>
</tr>
</tbody>
</table>

Table 2 demonstrates the preparation of the antibody reagents which will be used during this example. All of these reagents must be diluted 1:10 with Specimen Diluent or Wash Buffer prior to use (see Table 2):

Table 2: Antibody Preparation

<table>
<thead>
<tr>
<th></th>
<th># wells</th>
<th>volume per well</th>
<th>Reagent + Diluent</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMLCR</td>
<td>1</td>
<td>50 ul</td>
<td>5 ul reagent</td>
<td>Measures Class I and Class II binding</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>45 ul specimen</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>diluent</td>
<td></td>
</tr>
<tr>
<td>SA-PE</td>
<td>1</td>
<td>50 ul</td>
<td>5 ul SA-PE</td>
<td>Detect LMLCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ 45 ul wash buffer</td>
<td></td>
</tr>
<tr>
<td>Conjugate</td>
<td>4</td>
<td>50 ul</td>
<td>20 ul anti-human IgG</td>
<td>Detect human IgG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+180 ul wash buffer</td>
<td></td>
</tr>
</tbody>
</table>

1. LMLCR: Lysate Control Reagent. Biotinylated monoclonal antibodies specific for HLA Class I and HLA Class II. This will be added at the same time patient and control sera are added, in a separate well to determine the amount of donor HLA capture.
2. SA-PE Conjugate: Streptavidin-phycoerythrin conjugate which will be added to the LMLCR control well during the antibody detection step.
3. Conjugate: PE conjugated anti-human IgG, which will be added during the antibody detection step to detect bound human IgG.

Once prepared, keep all reagents in the dark. They are all light-sensitive.

Phase I: Bead Preparation (using Table 1 as an example)

1. Determine the number of assay wells that will be required. In the above example (Table 1), 4 assay wells will be used. One additional well was included to allow for losses due to pipetting.
2. Vortex the Capture beads.
3. Mix the capture beads with the appropriate volume of lysate in a polypropylene snap-cap tube. In the example shown in Table 1, 25ul beads are mixed with 40ul lysate.
4. Incubate the lysate/beads mixture for 30 mins at room temperature on a rotator. Protect the lysate/bead tube from exposure to light, by wrapping in foil, or covering with a box.
5. After the 30 min incubation, add the volume of Wash Buffer calculated from Table 1. In this example, 210 ul Wash Buffer will be added. Vortex thoroughly. The beads are now ready to be added to the assay microwell.
6. Add 150 ul distilled water to each well of the filter plate that will be used. After 2-5 mins, aspirate the buffer from the plate by using the vacuum manifold.
7. Transfer 55ul of each diluted lysate/bead combination (in this example we are only using one lysate/bead combination) to each assay well.
8. Add 100ul of Wash Buffer to each well. Aspirate.
9. Add 250ul of Wash Buffer to each well. Aspirate. Repeat two more times for a total of three 250ul washes.

**Phase 2: Addition of sera to the assay plate**

1. Add 50ul LMLCR to the designated well (see example in Table 2).
2. Add 38ul Specimen Diluent (blue solution) to each test well. In this example, three wells will be tested: the laboratory’s negative control serum, the laboratory’s positive control serum, and the patient serum.
3. Add 12ul of the appropriate serum to the appropriate well.
4. Cover the test wells with an adhesive plastic sealer.
5. Protect the sealed plate from light by using foil or a cover box.
6. Incubate for 30 minutes at room temperature in the dark on a rotating platform (approx. 200 rotations per minute).
7. Following the 30 minute incubation, carefully remove the plastic adhesive.
8. Add 100ul Wash Buffer to each assay well.
9. Mix by tapping the plate and aspirate the plate on the vacuum manifold.
10. Add 250ul Wash Buffer to each well, aspirate, and repeat two more times for a total of three 250ul washes.

**Phase 3: Addition of Secondary Conjugated Antibodies (see Table 2)**

1. Add 50ul of diluted SA-PE to the LMLCR well.
2. Add 50ul of diluted PE-conjugated anti-human IgG to each of the test wells (there are three in this example).
3. Cover the wells with adhesive plastic.
4. Protect from light by using foil or a cover box.
5. Incubate for 30 minutes at room temperature in the dark on a rotating platform (approx. 200 rotations per minute).
6. Following the 30 minute incubation, carefully remove the adhesive plastic.
7. Add 150ul Wash Buffer to each well, but DO NOT ASPIRATE on the vacuum manifold.
8. Mix the well contents by tapping the plate.
9. Collect data with the Luminex instrument using the manufacturer’s recommendations.

Calculation/Interpretation of Results

The Luminex instrument provides raw data as Mean Fluorescent Intensities (MFIs). Software is provided to interpret the MFIs relative to the 3 control beads provided in each assay mix (see Figure 1). In addition, each lot of beads has been tested by the manufacturer using 30 individual donor lysates/30 negative sera obtained from 30 nonsensitized individuals to derive a Background Adjustment Factor (BAF). The BAF is an MFI value to compensate for the background noise in each lysate/sera combination. The BAFs for each sample are calculated by using the lot-specific equations indicated on the Recording Sheets provided with each kit. Because of this, DO NOT MIX REAGENTS FROM DIFFERENT LOTS.

Figure I

The 7 bead set includes control beads to monitor assay performance. There are three control beads used to measure nonspecific “background” IgG binding. These beads are used to normalize the signal of the MHC I and MHC II capture beads.
Since there are two different conjugates used in different wells during each assay (SA-PE and PE-anti-human IgG) there are two control beads to insure that the appropriate conjugate was used in each assay well. One bead (called probe 77) is conjugated with human IgG and will indicate that the PE-anti-human IgG has been added to that well. One bead (called probe 78) is conjugated with a biotinylated protein and will indicate that the SA-PE has been added to the well.

Table 3 shows the results from an assay which included 4 assay wells:

1) LMLCR control demonstrates a high MFI and low background binding indicating good capture of donor MHC I and MHC II antigens. In addition, a high MFI with Probe 78 and a low MFI with Probe 77 demonstrates that the correct conjugate (SA-PE) was used.
2) Laboratory’s Positive control serum demonstrates a high MFI for both Class I and Class II capture beads
3) Laboratory’s Negative control serum demonstrates a low MFI with both Class I and Class II capture beads
4) Patient sample demonstrates a positive anti-donor Class I response in the presence of a negative anti-donor Class II response

Note: High MFI reactivity with Probe 77 indicate that the appropriate anti-human IgG secondary was added to each of the positive control serum, negative control serum, and patient serum assay well.

Table 3: Sample Assay Results (MFIs)

<table>
<thead>
<tr>
<th>Lysate #37</th>
<th>Interpretation</th>
<th>Capture Beads</th>
<th>Background Control Beads</th>
<th>Conjugate Control Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Class I</td>
<td>Class II</td>
<td>CON1</td>
</tr>
<tr>
<td>Sample ID</td>
<td>Class I</td>
<td>Class II</td>
<td></td>
<td>CON1</td>
</tr>
<tr>
<td>LMLCR</td>
<td>17751</td>
<td>16003</td>
<td>14</td>
<td>34</td>
</tr>
<tr>
<td>POS</td>
<td>Pos</td>
<td>10964</td>
<td>10425</td>
<td>46</td>
</tr>
<tr>
<td>NEG</td>
<td>Neg</td>
<td>232</td>
<td>256</td>
<td>78</td>
</tr>
<tr>
<td>OSU83</td>
<td>Pos</td>
<td>Neg</td>
<td>19132</td>
<td>499</td>
</tr>
</tbody>
</table>
QUALITY CONTROL

In addition to the LMLCR, and laboratory's own positive and negative control sera run against each donor-specific MHC-coated bead (shown in Table 3), further QC is built into the DSA by the inclusion of Positive and Negative Control Sera matched to the provided Dried Lymphocyte Control included in the assay kit. These controls should be included with each test run to help determine if technical errors or reagent failures have occurred.

Expected results are:

1) The LMLCR control should display a high MFI and low background binding to indicate good capture of donor MHC I and MHC II antigens. In addition, Probe 78 should have a high MFI while Probe 77 should have a low MFI to demonstrate that the correct conjugate was used.

2) The Positive control supplied in the kit should demonstrate a high MFI for both Class I and Class II and the results should be interpreted as positive for antibodies to both Class I and Class II.

3) The Negative control supplied in the kit should demonstrate a low MFI with both Class I and Class II and the results should be interpreted as negative for antibodies to both Class I and Class II.
Applicable ASHI/UNOS/CLIA Standards:

A.4.2.2 Crossmatching
A.4.2.7 Other Applications
C.1.1.4 Proficiency Testing
D.4.6.17 Crossmatching
D.4.6.23.3
D.4.6.24.1
D.4.7 Performance Specifications
D.4.11.18 Crossmatching Controls

FREQUENTLY ASKED QUESTIONS:

1. What donor tissues may be used as source material for lysate preparation?

The most common sources of donor material are peripheral blood, spleen, and lymph node.

2. How many sera can be tested against any one donor?

The limiting factor in determining the number of patient sera that can be tested against a given donor is the amount of lysate obtained. From 80 cc blood, we have obtained 1-5 mls lysate. Each sample requires 8 ul lysate, therefore from 500 ul lysate, 40 crossmatches can be performed.

3. Are there sufficient numbers of Class II bearing cells present in peripheral blood to obtain a good yield of Class II molecules in the lysate?

To date, we have tested over 200 donors using pre-retrieval peripheral blood as source material, and have always obtained a good Class II “capture”.

4. Can the lysate be stored?

We have used lysates stored for over 7 years with good results.

5. Can the lysates be freeze/thawed?

We have used lysates that have undergone up to 5 cycles of freeze/thaw with no loss of Class I or Class II capture and no affect on strength of signal. We aliquot lysates (10/deceased donor) in 1 ml aliquots and store at -70 to -80 C.
6. Are you aware of any consistent discrepancies between specificity analysis and DSA crossmatch results?

The DSA often fails to detect antibodies with specificity to HLA-DQ and HLA-DP (see reference 4).

7. How sensitive is the DSA assay?

The sensitivity of the DSA assay is comparable to the sensitivity of the single antigen Luminex beads. In titration comparisons with flow cytometry crossmatching, the DSA often detects a higher dilution of antibody than the 3-color flow crossmatch.

Trouble Shooting and Commonly Encountered Problems

1. The assay employs 2 distinct secondary conjugates: PE-conjugated strepavidin (SAPE) and PE-conjugated anti-human IgG. The most common error when first using the assay is to add the wrong secondary to a particular assay well. This could result in a false negative. There are control beads which alert the user that this has occurred.

2. Establishing a cut-off. The software provided with the assay will result in many false positives, especially when the control beads demonstrate low MFI's. Each lab should determine its own cut-off method, analogous to how we have approached defining cut-offs for our other assays (e.g. flow cytometry).

3. Incomplete washing can seriously affect results.

4. Pipetting accuracy is important, especially since the dilution of patient sera is performed as part of the assay. Don't try to accurately pipet 8 ul patient serum with a 100ul pipetting device.

5. The negative control serum is positive!! See step 2. With regards to establishing a cut-off, the assay should be interpreted in light of all available specificity information and other crossmatch methodologies. We have found this assay to be invaluable in understanding flow B cell crossmatches which are unexpectedly positive.

6. Some patient sera will exhibit high background binding. This is precisely why the assay contains 3 control beads with which to monitor the background binding. Sera with extremely high levels of background binding will require some kind of treatment in order to lower the background so that the assay can be properly interpreted.
REFERENCES


Boldt, B, Ladvienka, K, Clarke, M, Chance, S, Donor-Specific Antibody Detection by Luminex, 20th EFI Immunogenetics and Histocompatibility Conference, Oslo, Norway, June 8-11, 2006