Section 2: Technical Advantages and Disadvantages of Serologic HLA Typing

Technical Advantages of Serologic HLA Typing

Serologic HLA typing can be an effective method to rapidly identify HLA antigen specificities in an individual’s HLA phenotype. This can be useful for the quick identification of the HLA Class I phenotype of an individual who needs HLA-matched platelet transfusion support, as a quick and less-expensive screen to identify serologically-HLA matched family members for bone marrow and hematopoietic stem cell (HSC) transplantation or for the rapid identification of specific HLA antigens associated with specific diseases. For smaller laboratories, the cost of performing serologic HLA typing can be similar to that of DNA typing, especially if inexpensive but reliable commercially-prepared typing trays are purchased with rabbit complement included.

Technical Disadvantages of Serologic HLA Typing

Serologic HLA typing can be a time-consuming and labor-intensive process to maintain in a busy laboratory that does not perform much other serologic testing. It requires significant reagent and equipment validation and periodic quality control, reagents for lymphocyte isolation, typing trays, rabbit complement, stain and fixatives and equipment such as microsyringes that may not be used for DNA or solid-phase, bead-based test systems.

With fewer laboratories screening for HLA antisera, the sources of good, consistently reliable antisera for in house typing trays or for purchase by manufacturers that prepare serologic typing are becoming fewer and fewer. This may prevent the clear and accurate definition of some HLA antigens where antisera are rare or reactivity is weak. While most HLA antigens can be identified by serology, it can be difficult to clearly identify certain HLA antigens (ex. A26 and A66) and some antigen combinations (ex. homozygous B35 vs. B35, 71 or B35, 72) depending on the antisera used. Certain HLA-C locus alleles (C*12, C*13, C*14, C*15, C*16, C*17, C*18) and DQA1, DPA1 and DPB1 alleles are not typed serologically due to a lack of available antisera. It is not possible to identify high resolution HLA alleles by serologic methods. These can be easily identified by DNA typing technologies.

With the advent of HLA typing using DNA methodologies, the development of high-throughput sequencing and the overall decreasing cost of performing DNA typing, serologic HLA typing has become less useful for many clinical applications. It is very important to rapidly and accurately identify HLA antigens in deceased donors for solid-organ allocation. In addition, the need for high resolution allele-level matching in HSC transplants precludes the use of serologic HLA typing. Many disease association tests as well as drug hypersensitivity or vaccine study eligibility require testing for specific high-resolution HLA alleles.
Section 3: Specimens and Materials

Reagents:

Isolated T Cell and B Cell Suspensions
HLA Class I Typing Trays
HLA Class II Typing Trays
Rabbit Complement, ABC (T Cell)
Rabbit Complement, DR (B Cell)
Stain/Quench Reagent
Eosin Y Dye
Formaldehyde
Light-Weight Mineral Oil (Saybolt Viscosity 158)
Phosphate-Buffered Saline (PBS)
0.9% Blood Bank Saline
Deionized or Distilled Water

Equipment:

Single- and Multi-Channel Microsyringes
Fluorescent Light Box or Lighted Work Surface
Permanent Markers
Plastic Containers (Urine Cups, Specimen Containers)
Weigh Boats
Timers
Glass or Plastic Transfer Pipettes
Glass Slides (2 x 3”)
Serologic Typing Trays with Corresponding Worksheets
Manufacturer’s Product Inserts
Ultra-Low (<-65°C) Freezer
Applicator Sticks
Absorbent Material (Gauze, Wipes or Paper Towel)
Complement Cooling Block
Complement Cups
Section 4: Procedure

Principles:

Serologic HLA typing is performed using the standard NIH microlymphocytotoxicity or complement-dependent cytotoxicity (CDC) assay. In this assay, very small amounts of well-characterized HLA antisera and viable lymphocytes are incubated together in wells of a microtiter plate. When known antibodies present in test serum recognize HLA antigen present on the surface of the lymphocytes, antibodies bind to antigen, forming antibody-antigen complexes. Rabbit complement with well-characterized reactivity is added to test wells followed by further incubation, which allows complement proteins to bind to antibody-antigen complexes present in the test well. This binding initiates the proteolytic cleavage of complement components in the complement cascade, resulting in a controlled cell lysis and death.

Following the completion of the complement incubation, cells are stained with vital stains such as Eosin Y or fluorescent stains, such as acridine orange (AO), carboxyfluorescein diacetate (CFDA) and ethidium bromide (EB). Once stained, a “fixative”, such as formaldehyde, sodium azide or EDTA stops the reactions and prevent further cell death. Completed test trays are visually examined using an inverted phase microscope and reaction scores are determined for positive and negative test reactions based on a standardized grading scale.

Procedure – Plating Serologic Test Trays:

A. Prepare the work area where samples will be plated by obtaining the following items:

1. Microsyringes to be used in testing.
   a. 1 ul single- or multi-channel microsyringes are used for the plating of cell suspensions and if used, the addition of Eosin Y.
   b. 5 ul multi-channel microsyringes: one for dispensing rabbit complement and one for dispensing stain/quench and fixative solutions.
   c. It is advisable to maintain dedicated single- and multi-channel microsyringes for the addition of cell suspensions and the various reagents used in testing. Dedicated microsyringes can help prevent the premature transfer of small amounts of stains and fixatives into test wells, which can weaken reactivity or cause false negative reactions.
   d. Microsyringes should be rinsed several times with 0.9% blood bank saline or PBS in preparation for plating. This will remove any excess water remaining after storage. Microsyringes will be emptied into a waste container just prior to their use in plating.
2. A permanent marker to record sample IDs on test trays.
3. Transfer pipettes. These can be glass or plastic, with or without an integral bulb.
4. Plastic ware: The size and selection is based on individual laboratory choice:
a. Complement cups may be used to hold cell suspensions, stain/quench or fixative solutions. They may also be used to keep thawed rabbit complement chilled in a cooling block or ice bath.

b. Weigh boats are an alternate choice for cell suspensions and other reagents used in testing.

c. Urine and small plastic specimen containers may be used to hold liquids used to rinse micro-syringes between sample and reagent additions.

d. A larger plastic container may be used as a temporary waste container for fluids used to rinse and clean microsyringes during their use.

B. **Obtain the isolated lymphocyte suspension to be plated.**

1. For Class I typing: isolated T cells or a mixed suspension of T and B cells.

2. For Class II typing: a suspension of isolated B cells.

3. Cell suspension should be maintained in a physiologic solution such as PBS, PBS with 0.6% sodium citrate, Hank’s Buffered Salt Solution (HBSS), RPMI or McCoy’s. Fetal calf/bovine serum (FCS/FBS) or pooled human serum (PHS) may also be added to help maintain good cell viability during testing.

4. The ideal lymphocyte suspension should be prepared at a concentration of ~2.5 – 3 x 10⁶ cells/ml.
   
a. Concentrations < 2.5 x 10⁶ cells/ml may yield an increased number of weak false positive reactions.

b. Concentrations > 3 x 10⁶ cells/ml may yield an increased number of false negative reactions.

5. The ideal lymphocyte suspension will have a pre-plating viability of > 95% however, samples with viability > 80% may yield acceptable test results. It is best to obtain new, fresh sample when pre-plating viability is < 80%, however, circumstances may prevent this. Caution must be taken when evaluating weak positive test reactions. Samples with poor viability must be evaluated and used at the discretion of laboratory staff.

6. The ideal cell suspension will not have excessive granulocyte, red blood cell (RBC) or platelet contamination that will interfere with reading and interpretation of testing results.

C. **Obtain typing trays to be plated from their frozen storage location and prepare them for plating.** Frozen storage at < -65°C is best for long-term storage of serologic HLA typing trays.

1. Allow trays to thaw at room temperature until all frozen antisera are thawed.

2. If possible, place trays onto a lighted work surface during this process to aid in thawing and allow wells to be checked for missing or insufficient antisera volumes.
3. Any wells found to be insufficient should be marked using a permanent marker. Place a mark on the underside of the tray to indicate potential false reactivity when trays are read.

4. Review the worksheet from the specific typing tray to identify the locations of negative, positive and cell-specific control sera. Identifying these locations prior to plating is important to help prevent carryover during plating.

5. Use a permanent marker to clearly label the thawed tray(s) with the appropriate identifier of the cell sample being plated. This can be sample name or unique identification number and computer-generated labels may also be used.

6. If trays are not plated immediately, they should be stored in a refrigerator at 2 - 8°C for up to 30 minutes. As a rule, trays should be thawed, labeled and plated immediately to reduce the risk of decreased reactivity that can occur when reagent antisera are maintained at room temperature.

7. The use of HLA typing trays containing monoclonal antibodies (monoclonal trays) is not described in this procedure. Refer to manufacturer's instructions available on the product insert or via the vendor's website.

D. Plating Cell Suspensions:

1. Obtain the cell suspension to be plated and gently swirl the sample tube to insure a homogeneous mixture. Fill the 1 ul microsyringe with the cell suspension being plated.
   a. If a single-channel microsyringe is used, place the needle tip into the cell suspension and rinse several times to insure a homogeneous mixture is present in the syringe for plating. Excessive mixing can reduce sample viability.
   b. When using a multi-channel microsyringe:
      i. Transfer the cell suspension into a complement cup or small plastic container. The remaining cell suspension may be returned to its original tube after plating or discarded.
      ii. Place the needle tips of the syringe into the cell suspension and rinse several times to insure a homogeneous mixture is present in the syringe for plating.

2. Confirm that the ID of the sample and the tray being plated match and plate cell suspensions on typing trays:
   a. Place the needle tips of the filled syringe into the first row and dispense 1 ul of cell suspension into each well.
      i. For 1 ul multi-channel microsyringes: One click of the syringe will add 1 ul of cell suspension to all wells of the row. Continue to click and add cell suspension to each successive row until the entire tray has cell suspension added.
      ii. For 1 ul single-channel microsyringes: It is common to plate in a serpentine manner, starting in well 1-A, continuing to 1-B through
1-F, then continuing down the next row 2-F through 2-A, up the next row, 3-A to 3-F until the entire 72-well tray is plated. Plating is performed in a similar manner with 60- and 96-well plates; up the odd-numbered rows and down the even-numbered rows.

iii. When dispensing cell suspensions into wells, the use of the soft-drop technique is recommended. Layering the cell suspension droplet into the oil while avoiding contact with the serum present can help prevent carryover of strongly-positive serum from one well to the next.

b. Confirm the location of negative and positive control sera to avoid carryover, especially from a positive control serum into the next well plated or into a negative control serum.

c. Wiping needle tips and/or click into an absorbent material, such as gauze, wipe or paper towel, to remove any excess positive serum that could result in carryover.

d. After cell suspension has been added to all test wells, gently tapping the tray on the work surface will promote mixing and encourage any unmixed cells and serum to mix together.

e. Examine each test well to insure all cell suspensions and sera are mixed. Cell suspensions appear to float in the oil present in the test well while test serum may be at the bottom of the well. If any wells contain cells and sera that are not mixed, any of the following steps may be taken to encourage mixing.

i. Use the pointed end of a syringe needle tip, paper clip, broken applicator stick or similar device to force the cell suspension to mix with serum. If a syringe needle tip is used, it should be very carefully wiped since a very small amount of serum can be pulled into the needle tip during plating. Wiping the device used or breaking off the used portion of applicator stick are other means to prevent potential carryover.

ii. To avoid physical contact with test wells:

a. Plated trays may be spin in a centrifuge using tray holders for approximately 30-60 seconds at 1000 RPMs. This will pull any unmixed cell suspensions into the bottom of the well to mix with antisera.

b. An electrostatic mixer may be used to mix cell suspension and antisera droplets.

E. Serum-Cell Incubation:

1. Place the plated tray on a flat work surface at room temperature. Trays may be stored covered or uncovered, at the preference of the laboratory.

2. Incubation times:
a. Commercially-prepared typing trays should follow the manufacturer’s recommended incubation times, which are included on the product insert for the specific tray.

b. For typing trays prepared in-house, incubation times should be based on procedures validated within the laboratory.

c. Commonly used serum-cell incubation times for the standard NIH microlymphocytotoxicity (CDC) assay are:
   i. For Class I typing trays: 30 minutes
   ii. For Class II typing trays: 30 - 60 minutes

F. Microsyringes used in plating should be rinsed with the rinse solution in use. Once a microsyringe will not be used in further testing, it should be rinsed with deionized/distilled water and returned to its storage locations.

G. Preparation and Addition of Rabbit Complement:

1. Remove the appropriate volume of rabbit complement for addition to the typing trays from frozen storage prior to the completion of the serum-cell incubation. Long-term storage of rabbit complement is best at < -65°C.

<table>
<thead>
<tr>
<th># of Wells to be Plated on Tray</th>
<th>Aliquot Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 – 72</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>96</td>
<td>0.75 ml</td>
</tr>
<tr>
<td>Two 60 or 72-well trays</td>
<td>1 ml</td>
</tr>
<tr>
<td>Two 96-well trays</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Four 60 or 72-well trays</td>
<td>2 ml</td>
</tr>
<tr>
<td>Four 96-well trays</td>
<td>3 ml</td>
</tr>
</tbody>
</table>

2. Frozen complement:

   a. Thaw according to the manufacturer’s product insert for commercially-prepared typing trays or for complement purchased for use with in-house typing trays.

   b. It is common to thaw rabbit complement at either room temperature or at 37°C. If complement is thawed at room temperature, it may be important to remove it from storage immediately after plating to allow sufficient time to thaw. If complement is thawed at 37°C, it should be removed 5 – 10 minutes prior to its addition to typing trays and before it is completely thawed.

3. Lyophilized complement:

   a. Prepare according to the manufacturer’s product insert.

   b. When reconstituting lyophilized complement, it can be helpful to allow it to thaw at room temperature for several minutes prior to the addition of water. This will allow it to more easily re-suspend once water is added.

4. Once thawed and prepared, rabbit complement must be stored either in a cooling block, in an ice bath or in the refrigerator until it is used. This will prevent the complement from losing any reactivity prior to its addition to typing
trays. Rabbit complement must be used within 30 minutes of thawing. Discard any remaining unused rabbit complement.

5. Fill a dedicated 5 ul multi-channel microsyringe with the rabbit complement for the tray being tested. Complement may be mixed with a pipette to obtain a homogeneous mixture however, care must be taken to avoid aeration and over-mixing, which can affect complement reactivity.

6. Place the needle tips of the filled syringe into the first row of the typing tray and dispense 5 ul of rabbit complement into each well of the row. Continue to add rabbit complement to each row until the entire tray has been completed.

H. Rabbit Complement Incubation:

1. Place the plated tray on a flat work surface at room temperature. Trays may be stored or covered or uncovered, at the preference of the laboratory.

2. Incubation times:
   a. Commercially-prepared typing trays should follow the manufacturers’ recommended incubation times, which are included on the product insert for the specific tray.
   b. For typing trays prepared in-house, incubation times should be based on procedures validated within the laboratory.
   c. Commonly-used rabbit complement incubation times for the standard NIH microlymphocytotoxicity (CDC) assay are:
      i. For Class I typing trays: 30-60 minutes
      ii. For Class II typing trays: 60 – 120 minutes

I. Staining and Fixing Test Reactions Using Eosin Y Dye and Formaldehyde:

1. Eosin Y dye (stain) and formaldehyde (fixative) have been commonly used in the standard NIH microlymphocytotoxicity assay when lymphocytes are isolated by density-gradient centrifugation.
   a. Eosin Y is commonly used as a 5% solution to stain lymphocytes in testing.
   b. Formaldehyde solution is commonly used to fix and preserve lymphocytes upon completion of the CDC assay. Formaldehyde concentrations ranging from 5 – 37% may be prepared to obtain a working solution with a pH of 7.2 – 7.4. The choice of concentration for use must be validated by the laboratory.

   Note: Formaldehyde is an aromatic organic compound that is known to be carcinogenic. Proper precautions must be taken when handling this chemical, which may include working under a hood and using a face mask. Glutaraldehyde is a less-toxic substitute for the use of formaldehyde.

2. Place a desired volume of Eosin Y dye into a small plastic container. 0.002 – 0.005 ml (2 – 5 ul) is added to each tray well. 0.2 – 0.5 ml (200 – 500 ul) is sufficient to complete one 60 or 72-well tray and 0.30 – 0.75 ml (300 – 750 ul) is sufficient to complete one 96-well tray.
3. Fill a dedicated multi-channel microsyringe with the desired volume of Eosin Y dye.

4. Place the needle tips of the filled syringe into the first row of the typing tray and dispense 2 – 5 ul of Eosin Y dye into each well of the row.

5. Set a timer for 5 minutes and continue adding Eosin Y dye to each row until the entire tray has been completed.

6. Place a desired volume of formaldehyde solution into a small plastic container. 0.005 ml (5 ul) is added to each tray well. 0.5 ml (500 ul) of formaldehyde solution is sufficient to complete one 60 or 72-well tray and 0.6 ml (600 ul) of formaldehyde solution is sufficient to complete one 96-well tray.

7. Fill a dedicated 5 ul multi-2-channel microsyringe with the formaldehyde solution.

8. After the 5 minute Eosin Y incubation is complete, place the needle tips of the syringe filled with formaldehyde into the first row of the typing tray and dispense 5 ul of the solution into each well of the row. Continue adding formaldehyde solution to each row until the entire tray is completed.

J. Staining and Fixing Test Reactions with a Stain/Quench Reagent:

1. Commercially-prepared products are available that contain a mixture of vital fluorescent stains, a fixative and a quenching agent.
   a. Acridine Orange (AO) and Carboxyfluorescein Diacetate (CFDA) are two vital fluorescent stains commonly used in stain/quench products. These stains identify negative (no cytotoxicity demonstrated as cell death) cells in testing.
   b. While Ethidium Bromide (EB) is more commonly used, EB and propidium iodide (PI) are both vital fluorescent stains that can be used in used in stain/quench products. These stains identify positive (cytotoxicity demonstrated by dead cells) cells in testing.
   c. Commonly used fixatives include ethylenediaminetetraacetic acid (EDTA) and sodium azide.
   d. Commonly used quenching agents include bovine hemoglobin and India ink.
   e. Stain/Quench reagents are most commonly used when cells are isolated using magnetic beads.

2. Place a desired volume of stain/quench reagent into a small plastic container or weigh boat. 0.5 ml (500 ul) is sufficient to complete one 60 or 72-well typing tray and 0.75 ml (750 ul) is sufficient to complete one 96-well tray.

3. Fill a dedicated 5 ul multi-channel microsyringe with stain/quench reagent.

4. Place the needle tips of the filled syringe into the first row of the typing tray and dispense 5 ul of stain/quench reagent into each well of the row. Continue adding stain/quench reagent to each row until the entire tray is completed.
5. Completed trays should have a tray lid or glass coverslip placed over the top of tray wells to prevent dust and debris from contaminating wells, making reading difficult.

6. Completed trays should be placed a flat surface to allow cells to settle into the bottom of the tray for approximately 30 minutes prior to reading. A faster alternative to help cells settle is to spin the trays in a centrifuge with tray holders for 1 minute at ~2000 rpms.

**Limitations:**

Serologic HLA typing is limited by its resolution and the availability of good reagent antisera. Most HLA-A, B, DR and DQ antigens can be identified by serology. Null alleles, some C locus antigens and DQA1, DPA1 and DPB1 alleles cannot be identified by serology due to a lack of available antisera. Serologic HLA typing will give fairly comparable results to low resolution HLA typing performed by DNA methods, however, some HLA antigens and antigen combinations are best identified by DNA methods.
Section 5: Interpretation, Reporting and Troubleshooting

Interpretation of Serologic Typing Results:

All serologic typing trays have specific requirements for controls and antisera that are defined by ASHI Standards. These requirements are specific to the type of tray used in testing: Class I typing trays with T cells vs. Class II typing trays with isolated B cells or a mixture of T and B lymphocytes. Test trays are read on an inverted phase microscope and results scored according to a standardized grading system, such as the ASHI scoring system.

A. Negative Control:

1. The negative control is used to determine the post-testing sample viability as a baseline in both Class I and Class II typing trays. It will also demonstrate that nothing was added to the test system has caused excess cytotoxicity.

2. Negative control sera are frequently referred to as pooled human serum (PHS) or normal human serum (NHS) and may be diluted with reagents such as RPMI-1640, McCoy’s media, Hanks Buffered Salt Solution (HBSS) or PBS prior to their use.

3. Negative control serum is routinely prepared from non-alloimmunized individuals, most commonly from blood group AB male donors with no history of sensitizing events such as transfusion.

4. During extensive quality control, negative control sera are tested to demonstrate the following important characteristics:
   
   a. The serum has no HLA antibody reactivity.
   b. It has no inherent cytotoxicity of its own.
   c. The serum relies on the addition of rabbit complement to the test system and not any endogenous complement activity.

5. Interpretation:
   
   a. The viability of the negative control well will routinely be \( > 80\% \), indicating acceptable negative test results.
   
   b. If the viability of the negative control well is \( < 80\% \), post-testing sample viability may make it difficult to clearly identify critical weak positive reactions from the test background. Poor viability also generates background staining that can make reading test results difficult.

B. Anti-Lymphocyte Positive Control:

1. The anti-lymphocyte positive control is used on both Class I and Class II typing trays to demonstrate that the test system is reliant on the addition of rabbit complement and the strength of reactivity has not been compromised in any manner.

2. The positive control may be prepared from a pool of carefully selected, highly reactive sera or as a monoclonal reagent specific to T and B cells.
3. Interpretation: The positive control well must have a strong positive reaction score of 8 or a positive reaction score of 6 for results to be considered valid.

C. Anti-T Cell Positive Control:
1. The anti-T cell positive control is used on Class II typing trays to demonstrate the amount of contaminating T cells present in the B cell suspension used for testing. Contaminating T cells are problematic since they do not have Class II antigens expressed on their surface and will make positive reactions appear more weakly positive.
2. Anti-T cell positive control serum is routinely prepared as a monoclonal reagent serum that demonstrates reactivity against T cells but not B cells.
3. Interpretation: The anti-T cell positive control must have a negative reaction score of 1 or a doubtful negative reaction score of 2 for test results to be valid.

D. Anti-B Cell Positive Control:
1. The anti-B cell positive control is used on Class II typing trays to demonstrate the amount of B cells present in a B cell suspension used for testing. If an insufficient amount of B cells is present in the cell suspension, valid results may not be obtained.
2. Anti-B cell positive control is routinely prepared as monoclonal reagent serum that demonstrates reactivity against B cells but not T cells.
3. Interpretation: The anti-B cell positive control must have a strong positive reaction score of 8 or a positive reaction score of 6 for test results to be considered valid.

E. Anti-Granulocyte and Anti-Monocyte Positive Controls:
1. Some manufacturers include anti-granulocyte and anti-monocyte positive control sera on their trays as a means to identify these contaminating phagocytic cells. Both types of cells will phagocytize vital stains present in the test system, yielding weak false positive reactivity and typing trays that can be difficult to read and interpret.
2. Anti-granulocyte and anti-monocyte positive controls are routinely prepared as monoclonal reagent sera that demonstrate specific reactivity against either monocytes or granulocytes.
3. Interpretation: Both types of positive controls should have a negative reaction score of 1 or a doubtful negative reaction score of 2 for test results to be valid.

F. Interpreting Typing Results – Class I Typing Trays:
1. Once test scores have been recorded on typing tray worksheet(s) for the sample tested and all controls have been reviewed for validity, observe the clusters of positive reactions to identify the HLA Class I antigens present. Once identified, record these antigens in the space provided on the tray worksheet.
2. As a rule of thumb, there should be at least 2 operationally monospecific or 3 operationally multi-specific, non-overlapping positive sera to serologically define an HLA Class I antigen.
For example:

<table>
<thead>
<tr>
<th>Serum</th>
<th>Specificities</th>
<th>Reaction Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abe</td>
<td>A1</td>
<td>8</td>
</tr>
<tr>
<td>Beth</td>
<td>A1, A3, A11, A36, A80</td>
<td>8</td>
</tr>
<tr>
<td>Claude</td>
<td>A1, A36, A80</td>
<td>8</td>
</tr>
<tr>
<td>David</td>
<td>A1, A36</td>
<td>8</td>
</tr>
<tr>
<td>Ellis</td>
<td>A3, A11</td>
<td>8</td>
</tr>
<tr>
<td>Frank</td>
<td>A11</td>
<td>8</td>
</tr>
<tr>
<td>George</td>
<td>A11, B35</td>
<td>6</td>
</tr>
</tbody>
</table>

The test reactions above are sufficient to clearly identify both A1 and A11 in the cell being typed since there are at least 3 non-overlapping sera to define A1 (Abe, Claude and David) and A11 (Ellis, Frank and George). In this case, the reactivity of the serum, Beth cannot be clearly attributed to either A1 or A11.

This is not the case with the following reactivity:

<table>
<thead>
<tr>
<th>Serum</th>
<th>Specificities</th>
<th>Reaction Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abe</td>
<td>A1</td>
<td>8</td>
</tr>
<tr>
<td>Beth</td>
<td>A1, A3, A36, A80</td>
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</tr>
<tr>
<td>Claude</td>
<td>A1, A11, A36, A80</td>
<td>8</td>
</tr>
<tr>
<td>David</td>
<td>A1, A36</td>
<td>8</td>
</tr>
<tr>
<td>Ellis</td>
<td>A3, A11</td>
<td>1</td>
</tr>
<tr>
<td>Frank</td>
<td>A11</td>
<td>1</td>
</tr>
</tbody>
</table>

It is possible to clearly identify A1 since there is 1 operationally monospecific (Abe) and 2 multi-specific, non-overlapping (Beth and David) sera that are positive. Unless an additional HLA-A locus specificity is identified, it is not possible to clearly determine whether the individual is homozygous for A1 (inherited A1 from both parents) or whether A36 or A80 are also present in the phenotype. Further testing with sera that are able to clearly identify A36 or A80 without the presence of A1 reactivity would be necessary.

3. In addition to HLA-A and –B locus sera, Class I typing trays include antisera to identify reactivity to the HLA-B locus public epitopes, Bw4 and Bw6. HLA-B locus antigens contain either the Bw4 or Bw6 public epitope and correlating the B locus antigens present with the observed public epitope reactivity can be a useful identification tool. Bw4 and Bw6 reactivity must be reported when performing deceased donor typing for solid organ allocation prior to transplant.

G. Interpreting Typing Results – Class II Typing Trays:

1. Once test scores have been recorded on typing tray worksheet(s) for the sample tested and all controls have been reviewed for validity, observe the clusters of positive reactions to identify the HLA Class II antigens present. Once identified, record these antigens in the space provided on the tray worksheet.

2. As a rule of thumb, there should be at least 3 operationally monospecific or 5 operationally multi-specific, non-overlapping positive sera to serologically define an HLA Class II antigen.
For example:

<table>
<thead>
<tr>
<th>Serum</th>
<th>Specificities</th>
<th>Reaction Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>George</td>
<td>DR8, DR11, DR12, DR13</td>
<td>8</td>
</tr>
<tr>
<td>Harry</td>
<td>DR8, DR11</td>
<td>8</td>
</tr>
<tr>
<td>Ingrid</td>
<td>DR11</td>
<td>8</td>
</tr>
<tr>
<td>James</td>
<td>DR11</td>
<td>8</td>
</tr>
<tr>
<td>Kelly</td>
<td>DR11, DR12</td>
<td>8</td>
</tr>
<tr>
<td>Larry</td>
<td>DR11, DR12, DR13, DR14</td>
<td>8</td>
</tr>
<tr>
<td>Mary</td>
<td>DR11, DR12, DR13, DR1</td>
<td>8</td>
</tr>
<tr>
<td>Nelly</td>
<td>DR8</td>
<td>8</td>
</tr>
<tr>
<td>Ophelia</td>
<td>DR8, DR12</td>
<td>8</td>
</tr>
<tr>
<td>Patrick</td>
<td>DR8</td>
<td>8</td>
</tr>
<tr>
<td>Quince</td>
<td>DR8</td>
<td>8</td>
</tr>
</tbody>
</table>

The test reactions above are sufficient to clearly identify both DR8 and DR11 in the cell being typed since there are 3 monospecific DR8 sera to define DR8 (Nelly, Patrick and Quince) and 2 monospecific (Ingrid and James) and at least 3 non-overlapping (Kelly, Larry, Mary, Nelly) sera (total of 5) to define DR11.

3. In addition to HLA-DR and –DQ locus sera, Class II typing trays include antisera to identify reactivity to the HLA-, DR51, DR52 and DR53 antigens. Most HLA-DR locus antigens contain one of these epitopes and correlating the DR locus antigens present with them can be a helpful identification tool. DR51, DR52 and DR53 reactivity must be reported when performing deceased donor typing for solid organ allocation prior to transplant.

4. Since HLA-DR and DQ gene loci are located close to each other in the Major Histocompatibility gene complex (MHC), DR and DQ antigens tend to be inherited together in linkage disequilibrium. It can also be helpful to correlate the DR and DQ antigens that occur together in a Class II phenotype.

H. When only one HLA antigen is identified for any Class I or Class II locus:

a. It is not uncommon that the individual has inherited the same antigen from each parent and appears to be homozygous for the antigen.

b. While not as common, it is possible for an individual who appears to be homozygous for an antigen to have a less-common antigen or less serologically-reactive antigen present in their phenotype. For example, in the case of an individual who appears to be homozygous for A1, it may be possible that they could have A1, A80 or A1, A36 in their phenotype; well-characterized antisera are important and not all antisera may be screened with rarer antigens that may be found only in certain ethnic groups, such as A80.

I. When weak reactivity is present that is not explained by the antigens identified, it is possible this reactivity is due to crossreactivity. Crossreactivity occurs when antibody present in serum recognizes small amino acid sequences known as epitopes that may be shared among antigens. For example: when B7 is present in a typing, it is not uncommon to see weak reactivity with antisera that test negative with B7 but positive for antigens that are crossreactive (share an epitope) with B7, such as B81, B73, B42, B55, B60, B61, etc.

Reporting Serologic HLA Typing Results:
Results from serologic HLA typings should be reported based on the protocol of the laboratory however, the format that is frequently used is:

For Class I typings:  A1, 2; B8, 44  (Bw4, w6); Cw5, 7
For Class II typings:  DR4, 17  (DR52, 53); DQ2, 8

Refer to the latest version of World Health Organization (WHO) “Nomenclature for factors of the HLA system, 2010” (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2848993/) and the HLA Dictionary (http://www.ebi.ac.uk/ipd/imgt/hla/dictionary.html) for the latest information on HLA nomenclature.

Troubleshooting Serologic HLA Typing:

A. Background viability may be due to:
   1. Cell suspensions prepared too far in advance of testing
   2. Improper handling of cell samples: too vigorous centrifugation, improper pH or isotonicity of reagents. Overmixing cell suspensions while filling microsyringes create shearing forces that can damage cells.
   3. Patient state of health and treatment: patients with hematologic disorders, such as leukemia and lymphoma may have damaged or dead cells present or may have fragile cells in various states of maturation.
   4. The presence of toxic substances such as microbial toxins, detergents or solvents.
   5. Frozen lymphocytes may have reduced viability depending on their handling during cryopreservation and thawing
   6. The presence of xenoantibodies (rabbit anti-human) in rabbit complement. Using complement prepared from rabbits no older than 7 days will normally reduce human exposure and greatly decrease the presence of xenoantibodies. Pre-absorption of rabbit complement with splenic or leukemic blast cells may be advisable when performing testing with more sensitive leukemic cells.
   7. If background viability makes it difficult to interpret reactions and invalidates testing, it may be necessary to re-isolate and DNase treat lymphocytes, perform HLA typing by DNA or request a new sample be collected.

B. If the anti-lymphocyte positive control has a reaction score of 4 or less, the test results should be considered invalid and repeat testing should be performed.

C. If the anti-T cell positive control has a reaction score of 4 or more, the B cell suspension used for Class II typing contains too many T cells, which can make reading and interpreting weak positive reactions difficult. Test results should be considered invalid and re-isolation of additional B cells and repeat testing should be performed.

D. If the anti-B cell positive control has a reaction score of 4 or less, an insufficient amount of B cells is present in testing and test results are invalid. Re-isolation of additional B cell suspensions should be performed or if the lymphocyte amount in the sample is very low, it may be necessary to perform Class II typing by DNA or obtain additional sample.
E. If an anti-granulocyte or anti-monocyte positive control has a reaction score of 4 or greater, it may be necessary to treat the sample to remove the contaminating cells. Treatment may be performed with carbonyl iron or monoclonal-antibody preparations specifically designed to remove contaminating cells. Re-isolate additional B cell suspension and re-plate typing trays.

F. The absence of complement-mediated cell death may be due to several factors:

1. The absence or insufficient amount of antisera or complement present in the test well.
2. Complement or antibody inactivation due to protein denaturation, aeration, microbial contamination or protein precipitation in the presence of high ion concentration.
3. Improper incubation temperature: It is important to monitor room temperature to insure incubations occur between 20 - 24°C. Temperatures below 20°C can lower the reaction kinetics and reduce reactivity. High temperatures can result in degradation of thermolabile complement proteins.
4. Antigen excess caused by an excessive number of cells. It is important to visualize the amount of cells present in the cell suspension and adjust the suspension volume to obtain a cell concentration of ~ 2.5 – 3.0 x 10⁶ cells/ml.
5. Cells may have been prematurely exposed to fixative prior to the addition of complement. This may occur if microsyringes dedicated for specific reagents are not used.
6. Lymphocytes isolated from EDTA-anticoagulated blood may not be killed during the CDC assay without additional wash steps.

G. When only one antigen is detected in an HLA phenotype, the typing results should be closely examined for the possibility of an undetected antigen whose reactivity is masked by the presence of other explained positive reactivity. Additional serologic typing trays may be performed, however, DNA typing or family studies may be necessary to clearly identify the serologically-defined antigen.
Section 6: Validation Essentials

A. Many of the reagents used in serologic HLA typing require initial validation and lot-to-lot or shipment-to-shipment quality control to insure their proper function in testing. These reagents include:

1. Rabbit complement: Since serologic HLA typing is a complement-dependent assay, extensive initial validation is important to identify an appropriate lot of complement for use with typing trays. Characteristics taken into account when selecting new complement should include reagent that provides optimal reactivity based on known HLA antibody specificities of test antisera with little to no non-specific reactivity at an optimum working dilution.

2. Vital stains, fixatives and stain/quench reagents: The choice of vital stains, fixatives or stain/quench reagents is the choice of the laboratory based on the needs of their testing however, these reagents require initial validation as part of the serologic HLA testing protocol to insure their proper function.
   a. Stains must clearly differentiate negative and positive cells to ease the decision-making process when reading and grading serologic test reactions.
   b. Fixatives must preserve reactivity as it appears upon completion of testing and prevent further cell death, allowing the user to review test trays several hours up to a few days later when stored properly.
   c. Stain/quench reagents must combine the characteristics of both stains and fixatives in one reagent.

B. Microsyringes used in plating serologic typing trays require periodic validation to confirm their proper function in dispensing microliter volumes of cell suspensions and reagents. Commonly used methods to validate microsyringes include:

1. A gravimetric method where specific volumes of water are dispensed from the syringe into a container, multiple weights are determined and averaged and calculations convert the water weight to volume based on the specific gravity and temperature.

2. Acid-base titrations with specific volume settings are another method to verify the dispensing volume of microsyringes.

C. Typing trays require extensive validation when selecting trays from a commercial manufacturer. Typing tray validation should challenge the tray’s ability to identify a broad range of antigens without excess crossreactivity, non-specific reactions or result in poor viability due to excess cytotoxicity. The validation should include a sufficient number of samples to cover the range of antigens, highlighting specificities known to be problematic within the laboratory, those that are strongly reactive vs. weakly reactive and rare antigens, whenever possible.

D. Quality assurance processes within the laboratory should monitor how effective a lot of serologic typing trays are at identifying WHO-defined HLA antigen specificities.

1. This can be important when purchasing typing trays from manufacturers since using a minimum number of trays to perform testing and obtain accurate, timely HLA typing results. When a typing tray does not properly identify antigens, additional testing is required that delays reporting results and increases the cost of testing.
2. This is also important when a laboratory prepares its own home-brewed typing trays. Identifying problematic antisera allows for improvement processes such as re-defining antisera to accurately reflect the specificities the serum is currently able to define or replacement with a suitable serum of similar specificity.

E. External proficiency testing allows the laboratory the opportunity to monitor their ability to test for and identify serologically-defined HLA antigens with their current test protocols and typing trays.

F. Internal competency testing allows the laboratory the opportunity to monitor the ability of staff to test for and properly identify the HLA phenotype of previously tested, well-characterized lymphocyte samples.
Section 7: Clinical Considerations

The decision to perform serologic HLA typing must be based on the laboratory’s needs and the level of clinical support it provides to the programs it supports. Bone marrow/HSC transplantation requires high resolution, allelic-level HLA typing to identify allele-level matching family members or unrelated individuals as potential transplant donors. Serologic HLA typing in these circumstances is limited to antigen-level HLA typing that is able to quickly identify potential matching family members for further high resolution typing. Solid-organ transplantation requires antigen-level typing by molecular methods and serologic HLA typing is suitable only as a backup confirmatory method. Many disease associated-HLA antigens and HLA associated with drug hypersensitivity require high resolution allele-level typing however, HLA-B27 typing for ankylosing spondylitis is one of the more common disease association tests that can easily and rapidly be performed by serologic HLA typing. Serologic HLA typing allows the laboratory to rapidly perform HLA typing on patients who may need immediate and future HLA-matched platelet support as they undergo treatment.
**Section 8: Challenges and Future Directions**

With the changing clinical needs of the histocompatibility and transplant communities and the advent of higher-throughput DNA test methods that provide high resolution HLA typing at decreasing costs, laboratories have been required to perform HLA typing by DNA. This is slowly eliminating the need for and the clinical effectiveness of serologic HLA typing. As long as HLA antisera of reasonable quality are available to commercial manufacturers and there is sufficient demand for serologic HLA typing trays, commercial manufacturers will continue to support serologic HLA typing. Some laboratories maintain a frozen serum inventory in order to prepare in house serologic HLA typing trays as a cost-effective means to perform HLA typing.