Section 2: Technical Advantages and Disadvantages

Technical Advantages and Disadvantages of a Standardized Scoring System:

A standardized grading or scoring system is required by ASHI standards when analyzing reactivity seen in serologic HLA typing, HLA antibody testing and T and B cell crossmatches. The use of the ASHI scoring system, described in this module, is recommended. A standardized scoring system should be designed to reflect the range of positive reactivity seen with microlymphocytotoxicity assays in relation to the amount of cell death seen in testing. The use of a standardized scoring system can be helpful with several technical and clinical aspects of serologic testing:

A. Since cell viability is a very important indicator of test reactivity in microlymphocytotoxicity assays, the means to assess post-testing lymphocyte viability is essential. A baseline measurement of negative test reactions is commonly based on the results of the negative control.

B. As a mechanism to assess the amount of cell death resulting from cytotoxic damage in testing, a scaled increase in the amount of cell death in comparison with the negative control indicates the degree of positive reactivity, which has important clinical and technical ramifications:

The clinical implications of assessing the degree of positive reactions include:

1. In HLA antibody testing and crossmatches, the presence of HLA antibodies helps determine:
   a. Whether positive crossmatch results indicate donor-specific antibody reactivity that may result in antibody-mediated graft rejection following transplantation.
   b. Which unacceptable HLA antigens to avoid and mismatched antigens that may be acceptable during donor selection for solid organ transplantation and for HLA-matched platelet transfusion.

2. In serologic HLA typing, known HLA antibody specificities of typing antiserum help identify the specific HLA antigens present in the test subject’s phenotype.

The technical implications of a standardized scoring system for positive reactions include:

1. The range of positive and negative test reactivity indicate the strength and specificity of antibody-antigen binding in serologic testing.
   a. In HLA antibody testing, this is important for the identification of antibody specificities in patient serum samples and reagent antisera.
b. In crossmatches, this is important as an in vitro indicator for successful transplantation.

c. Since serologic HLA typing depends on strong, consistent antisera with well-characterized specificity, monitoring reaction scores can be an important part of laboratory quality control and quality assurance systems. It can be helpful to assess whether commercially-prepared trays have good ability to identify problematic HLA antigens. With home-brewed typing trays, it is important to monitor antisera to assess the need for replacement or re-definition of specificities.

2. The ability to monitor tech-to-tech variation as part of both training, technical competency and quality assurance systems.

3. The ability for inter-laboratory comparison of test results, as needed, which can be important with proficiency testing.

Technical Advantages and Disadvantages of Microscope Systems:

Serologic testing is performed in trays with test wells that have depth, which accommodates the volume of reagents added but allows the cells to settle into many different planes, including along the sides or edges and in the bottom of the well. This multidimensional test well does not allow the user to read test results with a standard bright-light microscope and requires the use of a more specialized, inverted phase-contrast microscope.

The type of inverted, phase-contrast microscope used is dependent on the reagents used to perform serologic testing. If Eosin Y dye is used to stain cells to visualize positive and negative cells, a bright-light inverted phase microscope should be used. The addition of Eosin Y dye leads to darkly-stained positive cells against a dark-red background while negative cells stain similar to the background of the well. If fluorescent vital stains are used, an inverted phase fluorescent microscope with mercury vapor lamp must be used. An inverted phase fluorescent microscope is significantly more expensive than a bright-light inverted phase microscope with the addition of lamp housing and filters to accommodate the mercury lamp. An additional expense is incurred with the use of mercury vapor lamps, which require frequent replacement to maintain an optimally bright fluorescent light source. While a fluorescent microscope system is more costly, the results visualized are much easier to interpret since the fluorescent stains provide clear, distinct color differences between the populations of negative and positive cells.

Technical Advantages and Disadvantages When Reading Test Trays:

Reading serologic test trays can be labor-intensive, especially when there are many trays to be read and reaction scores to be documented manually. A completely manual system requires the user to manually move the microscope stage from well to well through the rows of the tray while manually recording reaction scores onto corresponding test worksheets.

Automated reading systems can be somewhat expensive but their use is more efficient than manual methods. These systems allow the user to simply press the appropriate buttons to record reaction scores into software while programming directs the automated microscope stage to move the tray through the series of wells to be read. The automated stage system
normally has programming that allows the entry of specific test information. For typing trays, lot-specific tray information, such as lot number and expiration date, antisera names/IDs, HLA antibody specificities and well locations can be entered ahead of time and save for future use. When a tray is scored by the user, the automated system can print a worksheet complete with sample and test dates, technologist name/initials, information on reagents used, technique performed, tray lot information and reaction scores. An automated system is an initial expense, however, it can be a time-saving, invaluable tool for laboratories that perform a large volume of serologic testing.
Section 3: Specimens

Lymphocytes that are isolated from whole blood or tissue samples and tested in the complement-dependent cytotoxicity (CDC) assays are incubated in several steps with multiple reagents, including antisera, complement, anti-human globulin, vital stains and a fixative.
**Section 4: Procedure**

**A. Samples and Equipment:**

- Inverted, Phase Contrast Microscope with 10X Objective Lens OR
- Inverted, Phase Contrast Fluorescent Microscope with 10X Objective Lens and Mercury Vapor Lamp
- Completed Serologic Test Trays
- Automated Tray Reading System (Automated Microscope Stage with Computer Software)
- Laboratory Computer
- Corresponding Test Tray Worksheets

**B. Reading Serologic Test Trays**

1. Power up the inverted, phase contrast microscope according to the manufacturer’s operating instructions and allow the lamp to warm up to obtain maximum function and brightness. Bright-light microscopes may need only a few seconds however, mercury vapor lamps on fluorescent microscopes may take several minutes to obtain maximum brightness.

2. Obtain the completed serologic test tray and place onto the inverted, phase-contrast microscope’s stage. Many microscopes are equipped with tray holders that are a similar size to microtiter trays used in HLA testing. Automated tray reading systems frequently have tray holders that move the tray based on computer-assisted commands.

3. Using the microscope controls, position the tray to begin reading on well 1A (row 1, column A). Microtiter trays are commonly labeled with numbered rows (1 – 12 for 72 and 96-well trays and 1 – 10 for 60-well trays) and lettered columns (frequently A – F for 60 and 72-well trays and A – H for 96-well trays).

4. View the contents of the well through the oculars of the microscope.
   a. Minor adjustments of well position may be necessary to center the well within the field of view.
   b. Opening or closing the microscope’s condenser can allow the user to maximize the brightness of the field of view.
   c. With a fluorescent microscope, if the mercury vapor lamp is not fully centered in the field of view, it may be necessary to adjust the horizontal and/or vertical position of the lamp in its housing. Refer to the manufacturer’s operating instructions for further information.

5. Assess the amount of cell death present in each test well and determine the reaction score grading based on laboratory protocol. The use of the ASHI Scoring System is recommended.
6. Record the reaction score in the appropriate manner, either manually recorded in the appropriate space on the corresponding test worksheet or into the computer software that operates along with the automated stage in use. If an automated system is used, obtain a printout of the appropriate test worksheet.
Section 5: Interpretation/Reporting/Troubleshooting

A. Microscopic Appearance of Test Wells:

1. Eosin Y Dye/Fixative System:

   Using a bright-light inverted phase microscope, the background of a test well that contains Eosin Y appears dark red.

   Negative lymphocytes appear as small, regular round cells with well-defined cell membranes that appear the same color as the background of the test well.

   Positive lymphocytes appear as small, somewhat less-regular cells that are dark grey to black against the dark red background of the test well.

   Platelets appear as small translucent fragments that are the same color as the background of the test well.

   Granulocytic cells have the ability to phagocytize vital stains present in the test system. They are larger than lymphocytes and appear as irregular cells that are dark grey to black against the dark red background of the test well. It is possible to distinguish the multi-lobed nucleus of these polymorphonuclear (PMN) cells.

2. Fluorescent Vital Stains/Quenching Reagent System:

   Using an inverted-phase fluorescent microscope, the background of a test well that contains either acridine orange or CFDA and a quenching reagent appears a duller light greenish brown. Since immunomagnetic beads are commonly used to isolate lymphocytes within this test system, small, regular beads are present in the test well, loosely scattered in the bottom of the well or attached to lymphocytes.

   Negative lymphocytes appear as small, regular round cells with well-defined cell membranes that fluoresce bright green when either acridine orange or CFDA are used.

   Positive lymphocytes appear as small, somewhat less-regular cells that fluoresce reddish orange when either ethidium bromide or PI is used.

   Platelets appear as small translucent fragments that are the same color as the background of the test well.

   Granulocytic cells have the ability to phagocytize vital stains present in the test system. They are larger than lymphocytes and appear as irregular cells that fluoresce reddish orange. It is not usually possible to distinguish the multi-lobed nucleus of PMNs.
B. The ASHI Scoring System for Result Reporting:

The ASHI scoring system is a standardized numeric grading or scoring system that was developed to delineate negative, doubtful negative, weak positive, positive and strong positive test reactions. This system is based on the percentage of cell death observed upon the microscopic examination of test wells after completing serologic HLA testing. The ASHI scoring system is used for all types of serologic HLA testing, regardless whether testing is performed by the standard NIH microlymphocytotoxicity (complement-dependent cytotoxicity, CDC) assay, CDC assay employing the Amos wash technique or the anti-human globulin (AHG)-augmented CDC assay.

The negative control well must always be reviewed prior to assigning reaction score gradings. Depending on test design, this well is normally one of the first two wells read. This well contains the negative control serum, which has been extensively tested and shown to be negative in cytotoxicity testing. The viability of this well is indicative of post-testing lymphocyte viability and should be considered as a negative comparison. Any increase in viability over the negative control well may be considered positive, depending on the test circumstances and laboratory criteria.

<table>
<thead>
<tr>
<th>Reaction Score</th>
<th>% Cell Death</th>
<th>Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 – 10%</td>
<td>Negative</td>
</tr>
</tbody>
</table>

**Definition:** Little to no cell death has occurred as a result of antibody-mediated cytotoxicity. Post-testing sample viability is acceptable.

<table>
<thead>
<tr>
<th>2</th>
<th>11 – 20%</th>
<th>Doubtful Negative</th>
</tr>
</thead>
</table>

**Definition:** A small amount of cell death has occurred as a result of antibody-mediated cytotoxicity, however, this may also be indicative of poor sample condition and requires careful consideration. Some laboratories may consider this level of reactivity as positive for the purpose of identifying antibody-mediated reactivity in crossmatches and HLA antibody testing.

<table>
<thead>
<tr>
<th>4</th>
<th>21 – 50%</th>
<th>Weak Positive</th>
</tr>
</thead>
</table>

**Definition:** This amount of cell death caused by antibody-mediated cytotoxicity is sufficient to be considered weak positive in all test circumstances.

<table>
<thead>
<tr>
<th>6</th>
<th>51 – 80%</th>
<th>Positive</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>8</th>
<th>81 – 100%</th>
<th>Strong Positive</th>
</tr>
</thead>
</table>

**Definition:** This amount of cell death seen with an 8 reaction is the optimum amount of reactivity expected based on the dilution and known reactivity of both antisera and rabbit complement used within the test system. Commercial tray manufacturers and laboratories that prepare typing trays strive for test reactivity within this range.
Wells containing only cell debris with few to no intact lymphocytes is also considered a strong positive reaction. This may be indicative of excess cytotoxicity and should be interpreted with caution. This type of reactivity may indicate a serum-specific issue or may indicate excessive cytotoxicity due to complement reactivity.

0 Unreadable

Definition: It may not be possible to view the contents of the well or the amount of reactivity seen may be insufficient for valid interpretation of results.

C. Troubleshooting:

1. Test wells are frequently covered with a glass slide to prevent dust or debris from entering. This is also helpful to prevent evaporation of well contents, especially if trays are stored in a refrigerator to further review. How the glass slide is added to the tray can affect whether air bubbles are trapped into test wells, obscuring viewing the contents of the well.

   The use of a tray lid is also an acceptable substitute to prevent dust or debris and is less costly than the use of a glass slide however, it may not prevent evaporation of well contents or air bubbles.

2. Reagents used in testing may become contaminated with bacteria or yeast.
   a. Bacterial contamination may be sufficient to prevent viewing a sufficient amount of lymphocytes to determine a reaction score.
   b. Yeast are normally smaller than lymphocytes however, can be somewhat similar in size, making it difficult to identify negative lymphocytes vs. yeast.
   c. The presence of either type of microbe may also change the pH of the lymphocyte suspension, leading to positive test reactivity that is not the result of antibody-mediated cytotoxicity.
   d. Microbial contamination may result in invalid test results that necessitate repeat testing.
   e. It is recommended that antibiotics such as penicillin-streptomycin or gentamycin solutions be added to cell suspension media.
   f. Frozen storage for antisera and trays with thawing immediately before testing is also recommended to prevent microbial contamination.

3. Reagents used in testing may contain debris or crystalline matter:
   a. When formaldehyde or glutaraldehyde is used in testing, it is not uncommon to see crystal formation. It is important to filter any solution with either of these chemicals prior to use.
b. Eosin Y dye may also crystallize in solution, especially if it is not properly suspended during preparation. It is important to filter Eosin Y dye solutions prior to use.

c. Bovine hemoglobin used in quenching reagents may also contain debris or crystalline matter. It is important to filter solutions containing bovine hemoglobin prior to use.

d. It is also possible to find epithelial cells and hair present in test wells.

e. In most cases, debris or crystalline matter do not affect test results however, their presence can make reading difficult. If excessive, repeat testing may be necessary to obtain valid test results.

4. When fluorescent stains are used and faint test wells are visualized, check the number of lamp hours for the mercury vapor lamp in use in the microscope. Most power supplies have settings that allow the user to monitor the number of hours of lamp use. Mercury vapor lamps can maintain optimum brightness for anywhere from 100 – 1000 hours and due to their expense, should be closely monitored and replaced once brightness becomes questionable.

**Note:** When replacing a mercury vapor lamp, check facility, state and federal regulations regarding discard of the used mercury vapor lamp. Mercury is known to be a toxic chemical and should be handled according to OSHA Safety Standards.

5. Cell-serum ratio: Too many cells added to test wells can result in weak or false negative reactions. Too few cells per test well can result in inaccurate scoring or in the case of strong positive reactions, complete cell lysis.
Section 6: Validation Essentials

A. Quality Control and Quality Assurance with a Standardized Scoring System:

In addition to consistency in test result interpretation and reporting, the use of a standardized scoring system is important for consistent shipment-to-shipment, lot-to-lot and tech-to-tech comparison of the reagents involved in CDC testing. All reagent quality control, external proficiency and internal competency, and staff training protocols that involve serologic testing must employ a standardized scoring system to help minimize variation.

B. Quality Control of Microscopes:

All microscopes require quality control and maintenance procedures performed to maintain the instrumentation at optimal function. This may be performed by laboratory personnel, biomedical technicians within the facility or by trained service professionals. Refer to the operating manuals for the appropriate microscope for further details on quality control and maintenance procedures.

C. Quality Control and Validation of Automated Reading Systems:

Initial validation of an automated reading system should include verification of tray movement and the setup and operation of the computer-assisted software program that functions with the automated stage. Refer to the operating instructions for the automated stage system for further details on validation and quality control procedures.

Whenever a new version of computer software that operates the automated stage is implemented, re-validation is necessary to confirm proper tray movement and that the operation of the computer-assisted software program is acceptable.

D. Staff Training and Competency:

To prevent tech-to-tech variation, especially when new technologists are being trained, it is important to perform quality assessment of readings performed by laboratory staff. This can be as simple as all technologists reading the same test tray followed by review and discussion of any variations seen with all staff.

When a new technologist is being trained, it is important that the individual read a certain amount of test trays that are double-read by the laboratory supervisor or experienced technologist. This will help the new technologist gain a better understanding of any variations seen in reading while they are learning the standardized scoring system. This can be especially important when learning to interpret results that are very close to the negative/weak positive cutoff.
Section 7: Clinical Considerations

The use of a standardized scoring system that is well-validated among laboratory staff will help provide clear and concise test interpretations. Accurate scoring is important to identify the serologically-defined HLA antigens present in an individual's phenotype. It is also important to be able to distinguish negative and very weak positive test reactions that may implicate the presence of very weak but possibly significant donor-specific antibody, especially in a solid-organ transplant setting.
Section 8: Challenges and Future Directions

With the advent of high volume and high throughput DNA typing, solid-phase antibody testing and flow cytometric crossmatches, serologic testing is becoming a less-used technology in histocompatibility. While serologic test systems are still in use, it is important to be diligent about training staff in these test methods and maintaining the ability to read and interpret serologic test results with sufficient accuracy.