The presence of HLA antibodies can be detected by using a panel of well characterized lymphocytes in the microlymphocytotoxicity assay. The panel should be representative of the most common HLA antigens for the detection of sensitization and the possible further characterization of these antibodies.

Invitrogen™ HLA-FLT Trays are for use in the microlymphocytotoxicity assay for the detection and identification of Class I antibodies. Lymphocytes characterized for most of the WHO recognized HLA antigens are included. Invitrogen™ HLA-FLT Trays are 60-Well Cell Trays that contain 1 test consisting of 56 test wells. Two to five microliters of mineral oil are also added to each to prevent evaporation during freezing. Each well of the tray contains 2 µl of a specific lymphocyte preparation (6000 to 8000 mixed T and B lymphocytes/well) suspended in a freezing media of fetal calf serum and dimethyl sulfoxide in RPMI-1640. Four wells/test are reserved for the addition of positive and negative control sera. Cells in these wells are random duplicates of the panel and are designed to determine that the cytotoxicity assay is working, showing proper performance of the complement and expected results from designated incubation times.

CAUTION: HANDLE AS IF CAPABLE OF TRANSMITTING INFECTIOUS DISEASE
Human Lymphocytes from which this product was derived was tested and found to be non-reactive for Hepatitis B surface antigen (HBsAG) and Anti-HTLV-III as required by the FDA and by FDA approved methods of assay. However, no known test method offers complete assurance that human blood derived products will not transmit these diseases.

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1 **Kit Components:**

<table>
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<tr>
<th>Description</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 60 well cell tray contains 2 µl of a specific lymphocyte preparation</td>
<td>4</td>
<td>-55° C or colder in a NON-FROST FREE FREEZER</td>
</tr>
<tr>
<td>(6000 to 8000 mixed T and B lymphocytes/well) suspended in a freezing media</td>
<td></td>
<td>of fetal calf serum and dimethyl</td>
</tr>
<tr>
<td>of fetal calf serum and dimethyl sulfoxide in RPMI-1640, overlayed with 2-5 µl</td>
<td></td>
<td>sulfoxide in RPMI-1640, overlayed</td>
</tr>
<tr>
<td>mineral oil</td>
<td></td>
<td>with 2-5 µl mineral oil</td>
</tr>
<tr>
<td>1.2 Certificate of Analysis</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1.3 Worksheet</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

2 **Material, Reagents, and Equipment not Supplied:**

2.1 Dry vacutainer tube, Becton Dickinson & Co.
2.2 Centrifuge with a swinging bucket rotor, Beckman Instruments
2.3 Centrifuge, swinging bucket rotor, histoplate carriers - IEC Centra 7, Damon/IEC
2.4 Pasteur pipets, Curtin Matheson Scientific
2.5 1 ml plastic cryotubes or equivalent, Cryo Med
2.6 Microsyringes - 50 µl, 10 0µl, 250 µl, Matrix
2.7 Phase contrast microscope 150x, Nikon TMS or equivalent
2.8 Glass coverslide
2.9 Mineral Oil - Penreco, Drakeol® 10LT MIN OIL NF
2.10 Eosin Y - Solution 5%
2.11 12-37% Neutralized Formalin - pH 7.0 ±0.2
2.12 RPMI-1640
2.13 Pooled Human Serum (Heat Inactivated) - Invitrogen™ product code 34005100. Or Fetal Calf Serum (Heat Inactivated) - Sigma Chemical Co.
2.14 Positive and negative control sera - Invitrogen™ product code 1145005 and 3400005

3 **Sample Requirements:**

3.1 Using accepted medical procedure, collect whole blood without anticoagulant and allow blood samples to clot.
3.2 Centrifuge clotted blood at 2000 rpm for 10 minutes.

3.3 Remove serum and place in clean labeled vials.

3.4 If antibody screening is to be performed in this manner, prepare appropriate dilution(s) using RPMI-1640 containing 20% heat inactivated pooled human serum or heat inactivated fetal calf serum.

3.5 If screening is to be delayed, freeze undiluted serum and store at -55°C or colder.

4 Microlymphocytotoxicity Test Procedure:

The microlymphocytotoxicity assay uses lymphocytes as the target. This serologic assay measures cell death by the activation of complement (rabbit) in the presence of specific antigen-antibody combinations. The antibody-antigen complement reaction is measured by viewing the test microscopically at 150x magnification with phase contrast illumination using a vital stain such as Eosin Y or Trypan Blue. Dead cells will absorb the dye and exhibit appropriate color change. Negative cells remain viable, exclude the dye and do not exhibit any distinct nuclear detail.

4.1 Thaw serum samples, if necessary, and prepare appropriate dilutions if antibody screen is to be performed in this manner.

4.2 Remove Invitrogen™ HLA-FLT trays from freezer and allow to begin thawing slightly. Complete the thawing process by adding 5 µl prewarmed (37°C) wash medium (RPMI-1640 containing 10% fetal calf serum) to each well.

NOTE: Pooled human serum may be substituted for fetal calf serum.

4.3 Centrifuge trays at 1000 rpm for 60 seconds. Flick or aspirate the supernatant to remove.

4.4 Gently dispense 2 to 5 µl of mineral oil into each well to cover the cells.

4.5 Using an appropriate microsyringe, add 2µl of undiluted test serum or serum dilution(s) to each test well on the tray. In addition, negative control serum and positive control serum should be added to the appropriate wells.

    **Negative control in wells 1A, 1B**
    **Positive control in wells 10B, 10A**

4.6 Incubate the trays for 30 minutes at room temperature. (22°C±3°).

4.7 Using a 250 µl syringe, add 5 µl of rabbit complement to the wells, above the serum-lymphocyte mixture.

4.8 Incubate the trays for 60 minutes at room temperature (22°C±3°).

4.9 Using a 50 µl or 100 µl syringe, add 2 µl of filtered 5% aqueous Eosin Y to each well and incubate at room temperature (22°C±3°) for 3 to 5 minutes.
4.10 Using a 50 µl or 250 µl syringe, add 5 µl of filtered 37% neutralized formalin to each well.

4.11 Place a 2x3 inch coverslide over the tray and allow plates to stand at room temperature for at least 30 minutes before reading the reactions. Observe the test microscopically at 150x magnification using phase contrast illumination. It is recommended that the test be read within 48 hours.

5 **Interpretation:**

5.1 Dead cells (those possessing the antigens) absorb the dye and appear enlarged and darkened. Viable cells (those lacking the antigens) exclude the dye, appear brighter and smaller in size as compared to dead cells.

5.2 After correcting for percent dead cells in negative control wells, the test is graded as follows:

<table>
<thead>
<tr>
<th>% Dead Cells</th>
<th>Score</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>1</td>
<td>Negative</td>
</tr>
<tr>
<td>11-20</td>
<td>2</td>
<td>Doubtful negative</td>
</tr>
<tr>
<td>21-50</td>
<td>4</td>
<td>Weak positive</td>
</tr>
<tr>
<td>51-80</td>
<td>6</td>
<td>Positive</td>
</tr>
<tr>
<td>81-100</td>
<td>8</td>
<td>Strong positive</td>
</tr>
<tr>
<td>-----</td>
<td>0</td>
<td>Not readable</td>
</tr>
</tbody>
</table>

5.3 Identify antibodies using the standard antigram. Examine reaction pattern for positive reactions in cells that may have antigens in common. This will aid in distinguishing the specificities when recording test results.

6 **Performance Standards:**

6.1 Invitrogen™ Frozen Lymphocyte Panel has been extensively quality control tested.

6.2 The cells in each well have a minimum viability of 80%, with most wells exhibiting viabilities greater than 95%.

6.3 When using proper positive and negative control sera (such as manufactured by Invitrogen Corporation) the positive control wells will exhibit a 6-8 score and the negative control wells will exhibit a 1-2 score. These control wells are provided on each tray for testing complement activity and correct incubations, and not for determining cell viability.

6.4 Each lot of trays produced must meet a minimum standard by exhibiting acceptable performance against eleven known HLA antisera.

7 **Troubleshooting:**

7.1 Overall poor lymphocyte viability could be contributed to:
7.1.1 Room temperature is in excess of 26°C. Adjust room temperature to 22°C±3°C.

7.1.2 Failure to completely remove dimethyl sulfoxide (DMSO) from trays. Make sure all supernatant is removed before adding mineral oil to the wells.

7.2 Low lymphocyte count in the well could be contributed to:

7.2.1 Flicking tray with too much force or over-aspirating tray.

7.3 Overall weak reactions may be contributed to:

7.3.1 Room temperature is below 18°C. Adjust room temperature to 22°C±3°C.

7.3.2 The complement used has low activity.

8 Limitations and Precautions:

8.1 Cells on the Frozen Lymphocyte Panel are selected to represent most of the HLA antigens. However, because of the small panel size, antibody identifications against rarer groups are limited. Confirmation is recommended on a different panel.

8.2 Rabbit complement is a critical reagent in the lymphocytotoxicity test procedure. Complement provided with the Invitrogen™ HLA-FLT Trays must be used undiluted in this procedure. Invitrogen™ HLA-ABC complements have been quality control tested to meet performance criteria and exhibit <5% background toxicity (see catalog and/or complement package insert.) However, it is advisable that samples of each new lot of complement be tested in parallel with a known acceptable lot. A suitable rabbit complement should exhibit an 8 score with the positive control and most of the expected positive wells. It should exhibit a 1 score with the negative control and most of the expected negative wells.

8.3 Exposure to CO₂ should be avoided because of possible pH changes which may be anti-complementary.

8.4 Mineral Oil - It has been reported that some brands of mineral oil may be inhibitory in this assay. The brand listed in Section 2.9 of this instruction gives satisfactory results. Any other brands should be pretested prior to use.

8.5 Avoid excessive freeze-thaw cycles of serum samples as this may weaken antibody potency, possibly resulting in false negative reactions.

8.6 Serum should never be prediluted and then frozen. It is recommended that all dilutions be discarded at the end of the work day.

8.7 Screening with plasma must be avoided because:

8.7.1 The amount of anticoagulant may act as a diluent which weakens the potential antibody and may possibly cause false negative results.

8.7.2 Some anticoagulants, such as EDTA, inhibit the activation of complement, thereby causing false negative reactions.
References:


PR007
Revision 04
Print 7/06