

HLA Typing Tray

Class II (DR)

Product Insert

INTENDED USE

For use in the qualitative determination of HLA cell surface antigens using a complement-dependent microlymphocytotoxic technique.

For In Vitro Diagnostic Use.

SUMMARY AND EXPLANATION OF THE TEST

HLA characterization may be used for patient and/or donor assessment as applicable in Organ Transplant, Platelet Transfusion, Bone Marrow Transplant and Disease Association Studies.

GTI Class II (DR) HLA Typing Trays contain specific human antisera plus two positive and two negative controls. Well 1A, contains a murine monoclonal antihuman B lymphocyte antibody. The negative control serum, well 1B, is from a healthy non-transfused group AB male and has no cytotoxic reactivity. Well 12A contains a rabbit antihuman lymphocyte antibody as a positive control. This well must be positive in any valid test. Well 12B contains a murine monoclonal antihuman T-lymphocyte antibody. This antisera functions as another negative control with B lymphocytes. All antisera are characterized by serological testing using well-defined fresh and frozen cell panels. Multispecific sera are used only when a strong monospecific serum is not available.

PRINCIPLE OF THE PROCEDURE

Viable B lymphocytes are incubated with specific antisera and rabbit complement. If antigens present on the cell surface correspond to the antibodies in the sera, cell death will occur. Dead cells can be observed using phase microscopy after differential uptake of eosin dye or by 1 or 2 color fluorescence by differential uptake of Ethidium Bromide. Interpretation of the results is aided by the enclosed Sera Analysis sheet.

REAGENTS

- HLA Typing Trays: 72 typing sera with controls.
- Complement: Rabbit Complement, non-toxic to normal lymphocytes.
DR COMP-02
- Mineral Oil: All antisera in the tray are covered with mineral oil to prevent evaporation.

PRECAUTIONS

- All materials provided are to be used without dilution.
- Store trays at or below -65°C.
- Use trays before expiration date.
- Thaw trays at room temperature for 15 minutes and use within 30 minutes of thawing. Do not refreeze.
- Do not store trays in dry ice after sealed bag is opened.

CAUTION

- All human serum used in the preparation of the HLA Typing Trays have been tested and found negative for antibody to HIV, HCV, and HBsAg by FDA approved methods. No test method, however, can offer complete assurance that HIV, Hepatitis C virus, Hepatitis B virus, or other infectious agents are absent. Therefore, HLA Typing Trays should be handled in the same manner as potentially infectious material.
- Some of the sera contained on the trays may contain sodium azide as preservative. **WARNING:** Sodium azide reacts with lead and copper plumbing forming highly explosive metal azides. When discarded in a sink, the sink should be flushed with a large volume of water to prevent azide build up. Sodium azide is a poison and is toxic if ingested.
- Discard all components of this kit when completed according to local regulations.

SPECIMEN COLLECTION

Peripheral blood samples collected in Sodium Heparin, ACD or cells that have been cryopreserved using acceptable technique may be used for testing.

Sodium Heparin collection: 10cc of blood in 143 USP units of Sodium Heparin for magnetic bead isolation. 40-50cc of blood is required for nylon wool isolation. Heparinized blood should be kept at room temperature at all times prior to lymphocyte isolation and should be processed within 24 - 48 hours of collection.

ACD collection: 10cc of blood for magnetic bead isolation or 40-50cc blood for nylon wool isolation. ACD blood should be kept at room temperature at all times prior to lymphocyte isolation and should be processed within 24 - 48 hours of collection.

- Samples must be collected prior to patient myeloablative therapy.
- Collect samples prior to blood transfusion or at least 48 hours post blood transfusion.
- Excessive platelet contamination may mask the lymphocyte reaction or cause false negative reactions.

Lymph Node or Spleen tissue from organ donation may also be used. Process tissue and perform testing as soon as possible after tissue harvest.

PROCEDURE

The test procedure listed is our recommended protocol. The user may establish protocols with variations on the test procedure (e.g. fluorescence, incubation times, cell concentration); however, the user is responsible for determining protocols that are acceptable for use with the GTI HLA Typing Trays.

Materials Provided:

- HLA Typing Tray
- Recording Sheet
- Sera Analysis Sheet
- DR Complement

Additional Materials Required (User Provided):

- B Lymphocyte suspension in 5% Fetal Calf Serum in RPMI-1640.
- 0.05 mL syringe with needle in repeating dispenser (single or multiple) set to deliver 1 μ L.
- 0.25 mL syringe with needle in repeating dispenser (single or multiple) set to deliver 5 μ L.
- 1 mL syringe with a 6 nozzle head capable of delivering 3.3 μ L per nozzle in repeating dispenser.
- Cover slides: 75 X 50 mm.
- Eosin Staining Method
 - Eosin Y: 5.0 g per 100 ml HBSS. Filter through #1 Whatman filter paper.
 - Formaldehyde: Add 2 mL of 0.5% phenol red to 500 mL formaldehyde (10%). Adjust to pH 7.2 with concentrated HCl.
- Fluorescent Staining Method
 - Acridine Orange/Ethidium Bromide working solution (AO/EB).
 - 2% India Ink with 8% EDTA/PBS.
- Microscope
 - Inverted phase (Eosin Uptake Method).
 - Fluorescent (Fluorescent Uptake Method) with mercury vapor bulb and appropriate filters.

Test Procedure:

EOSIN DYE METHOD

1. Prepare B lymphocytes according to user laboratory procedures for lymphocytotoxicity testing.
2. Determine if cell viability is acceptable for test. Lymphocyte viability must be at least 85%.
3. Adjust cells to a desired concentration of $(1-1.5 \times 10^6 \text{ cells/mL})$ in 5% Fetal Calf Serum in RPMI-1640.
4. Remove tray from freezer and let thaw for at least 15 minutes at room temperature but not longer than 30 minutes.
5. Using a 0.05 mL syringe with needle attached, carefully add 1 μ L of a $1-1.5 \times 10^6 \text{ cells/mL}$ suspension of lymphocytes just under the oil. Ensure that cells and sera are adequately mixed.
6. Incubate for 45 minutes to 1 hour at 20-25°C.
7. Using a 0.25 mL syringe with needle attached to a repeating dispenser add 5 μ L of rabbit complement to each well.
8. Incubate for 1 - 2 hours at 20-25°C.
9. Add 3.3 μ L of 5% Eosin to each well using a microdispenser.
10. Allow eosin to penetrate the dead cells for 5 minutes.
11. Add 6.6 μ L of Buffered Formalin to each well to fix the reaction.
12. Allow cells to settle for 5-10 minutes before covering wells with a 75 x 50 mm coverslip.

13. Store trays, if not being read immediately, in the refrigerator prior to reading to reduce bubble formation.
14. Place the tray on an inverted phase microscope and examine each well at 100X magnification. Read the tray in the following serpentine pattern which corresponds to the Recording Sheet: 1A through 1F; 2F through 2A, etc.
15. Record reactions as observed on the Recording Sheet provided.

IMMUNOMAGNETIC BEAD METHOD with Fluorescence

1. Prepare B lymphocytes according to user laboratory procedures for lymphocytotoxicity testing.
2. Determine if cell viability is acceptable for test. Lymphocyte viability must be at least 85%.
3. Adjust cells to a desired concentration of $(1-1.5 \times 10^6)$ cells/mL in 5% Fetal Calf Serum in RPMI-1640.
4. Remove tray from freezer and let thaw for at least 15 minutes at room temperature but not longer than 30 minutes.
5. Using a 0.05 mL syringe with needle attached, carefully add 1 μ L of a $1-1.5 \times 10^6$ cells/mL suspension of lymphocytes just under the oil. Ensure that cells and sera are adequately mixed.
6. Incubate for 30 minutes at 20-25°C.
7. Add 20 μ L of AO/EB solution to each 1.0 ml of complement to be used and mix well.
8. Using a 0.25 mL syringe with needle attached to a repeating dispenser add 5 μ L of AO/EB complement solution to each well.
9. Incubate in the dark for 60 minutes at 20-25°C.
10. Add 3.3 μ L of india ink/EDTA solution to each well using a microdispenser.
11. Store trays in the refrigerator shielded from light for at least two hours but no longer than 48 hours before reading.
Note: No coverslip is required and bubble formation is not a concern with fluorescence method.
12. Observe reactions using a fluorescent microscope at 100X magnification. Read the tray in the following serpentine pattern which corresponds to the Recording Sheet: 1A through 1F; 2F through 2A, etc.
13. Record reactions as observed on the Recording Sheet provided.

Quality Control:

Specific Controls

- a) The B-cell positive control serum is in well 1A. This monoclonal antibody will cause cell death in only the B-lymphocyte population, and is an indicator of the purity of B-lymphocytes in the cell preparation. Other positive reactions should be compared to this control well in order to evaluate reaction strength.
- b) The negative control serum is in well 1B. There should be no cell death caused by the serum in this well. The “baseline” viability of the lymphocyte preparation can be determined by the reaction. Other reactions in the tray are scored by comparing the viability to the viability of the negative control which should be between 0-20% dead cells.
- c) The positive control serum is in well 12A. Valid tests should have at least 81-100% above background cell death in this well.
- d) The T-cell positive control is in well 12B. This monoclonal antibody will cause cell death in only the T-lymphocyte population and is an additional indicator of the purity of B-lymphocytes in the cell preparation.

INTERPRETATION OF RESULTS

Cell death should occur in any test well for which the cell surface antigen and the serum antibody are matched.

- Viewed using phase microscopy (eosin) live cells will appear bright and refractile, whereas dead cells will appear somewhat larger and stained dark with eosin dye. Eosin dye in the concentrations recommended in this procedure is effective only with phase-contrast microscopy. Without phase contrast the dead cells do not appear sufficiently dark to allow proper discrimination. Because swelling of the dead cells is increased by the addition of eosin, it is important to allow the dye to penetrate the dead cells for exactly 5 minutes prior to the addition of Formalin.
- Viewed using fluorescent microscopy (AO/EB) live cells will appear fluorescent green, whereas dead cells will appear fluorescent red.

Results are recorded using a grading system which corresponds to the percentage of dead cells (seen as large, dark and nonrefractile), within each well.

- 8 = 81-100% dead cells
- 6 = 51-80% dead cells
- 4 = 21-50% dead cells
- 2 = 11-20% dead cells
- 1 = 0-10% dead cells
- 0 = Not readable

Following recording of the results, compare the positive reactions to the specificities contained in each well. Positive reactions occur where antigens on the lymphocytes correspond to antibody present in the antisera. Identify the antigens which are present on the lymphocyte preparation being tested.

LIMITATIONS

Errors can occur at several stages. These are grouped below according to specific procedure.

a. Cell identification

When testing several samples simultaneously, the following switching errors may occur while isolating or testing:

- 1) interchanging cells
- 2) testing one cell twice while omitting another
- 3) mixing two samples during isolation

b. Cell isolation

The B-lymphocyte preparation must be as pure as possible. Several types of contamination problems are as follows:

- 1) Erythrocyte contamination can make microscopic evaluation difficult because of visual confusion with lymphocytes. Also, erythrocytes can deplete complement necessary for lymphocytotoxic reaction.
- 2) Platelet contamination can deplete antibody and complement, thereby causing false negative reactions.
- 3) Granulocyte contamination can cause false positives either by sticking to the tray when healthy or by increased sensitivity to rabbit complement. Granulocytes can also cause false positives due to phagocytosis of eosin.
- 4) T-lymphocyte contamination can cause false negative reactions, diminished reactivity, or non-reactivity. A good separation of T and B-lymphocytes is essential.
- 5) Cell concentration is important since the test is standardized using a certain antigen-antibody ratio. Cell suspensions of $\geq 2 \times 10^6$ lymphocytes may result in false negative reactions.

c. Adding the lymphocytes

Because the addition of lymphocytes to the wells can be done rapidly, several potential errors can occur:

- 1) Failure to mix lymphocytes with antisera (this is a common cause of negative reactions).
- 2) Skipping a well or row of wells.
- 3) Carryover of sera from one well to the next by the dispensing tips.

d. Microscopic Evaluation (“reading”)

This phase is susceptible primarily to errors of carelessness such as reading a tray in reverse order or recording errors. It is essential to have the phase-contrast microscope properly adjusted to view both dead and live cells.

e. Temperature

The microcytotoxicity test is a temperature dependent test. A temperature of 37°C is required to perform the test by the nylon wool isolation method and a range of 20°C to 37°C for the bead isolation method.

f. Complement

Rabbit complement must be nontoxic to normal lymphocytes. Careful handling of the complement is required. It must be completely thawed, mixed (gently), and kept cool continuously before using. DO NOT refreeze rabbit complement.

Bacterial contamination of the reagents or lymphocyte preparations may cause false positive reactions.

PERFORMANCE CHARACTERISTICS

Reproducibility studies have shown less than 2% errant reactions. Studies comparing HLA Typing Trays to DNA typing results showed 98.1% agreement. Sensitivity and specificity cannot be determined for the product as a whole. However the sensitivity and specificity of each typing serum included on the tray is provided in the Sera Analysis Sheet. Positive reactivity is defined as 50% or greater cell death caused by the antiserum. Parentheses indicate specificities which have been known to react with (~50%) of all cells possessing these antigens.

The Sera Analysis sheet is a summary of the results of testing the frozen panel described above with a panel of samples representing various ethnic individuals.

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