HLA Antisera
Instructions for Use
For In Vitro Diagnostic Use

The Invitrogen™ HLA Antisera are manufactured for the identification and definition of Histocompatibility Antigens and are for use in HLA typing.

The microlymphocytotoxicity assay uses lymphocytes as the target. Viable cells are easily extracted from peripheral blood, lymph nodes, spleen, etc. 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 150 x magnification with phase contrast illumination and a vital stain such as Eosin Y or Propidium Iodide. Dead cells (which possess the antigen detected by the specific antisera) will absorb the dye and exhibit an appropriate color change. Negative cells (those lacking the antigen detected by the specific antisera) remain viable and exclude the dye.

The Invitrogen™ HLA Antisera are for use in the microlymphocytotoxicity assay for definition of HLA antigens. They are specific for the HLA antigen(s) listed on the vial label. Each vial contains a specified volume of frozen or freeze-dried antisera containing 0.1% sodium azide as a preservative. These HLA Antisera are in vitro diagnostic reagents for professional use only and reactivity must be confirmed by the user with the specific complement that will be used in testing.

CAUTION: HANDLE AS IF CAPABLE OF TRANSMITTING INFECTIOUS DISEASE
Plasma and/or serum from which this product was derived has been tested and found to be negative for HBsAg, HIV-1, and HCV by an FDA approved method of assay. However, no test method offers complete assurance that HIV, hepatitis B virus, or other infectious agents are absent. They should therefore be handled as potentially infectious human blood specimens.

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1 **Kit Components:**

<table>
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<tr>
<th>Description</th>
<th>Quantity</th>
<th>Storage</th>
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<tr>
<td>1.1 Freeze-Dried Antisera containing 0.1% sodium azide as a preservative, 1 ml/vial</td>
<td>1</td>
<td>-20°C or colder in a NON-FROST FREE Freezer</td>
</tr>
<tr>
<td>OR Frozen Antisera containing 0.1% sodium azide as a preservative, 1 ml/vial</td>
<td>1</td>
<td>-55°C or colder in a NON-FROST FREE Freezer</td>
</tr>
<tr>
<td>1.2 Certificate of Analysis</td>
<td>1</td>
<td>------------------</td>
</tr>
</tbody>
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2 **Material, Reagents, and Equipment not Supplied:**

2.1 Bench top centrifuge
2.2 Light Microscope
2.3 Inverted Phase Contrast or Inverted Fluorescent Microscope
2.4 Pasteur pipettes 6 and 9 inch
2.5 Microtiter syringes 50 µl, 100 µl, 250 µl
2.6 12x75 mm and 17x100 mm test tubes
2.7 Hemacytometer
2.8 Glass Cover slide 2” x 3”
2.9 Magnetic beads, Invitrogen™ Dynabeads® HLA Cell Prep I (product code 219.02) and Invitrogen™ Dynabeads® HLA Cell Prep II (product code 219.03)
2.10 Micro Tissue Culture Plates, Invitrogen™ product code 71000100
2.11 5% Dextran
2.12 Ficoll-Hypaque solution
2.13 RPMI-1640 media with HEPES buffer and Pen/Strep added
2.14 Hanks Balanced Salt Solution (HBSS)
2.15 Trypan Blue
2.16 Eosin Y solution or CFDA
2.17 12-37% Neutralized Formalin pH 7.0±0.2 or Propidium iodide in quenching solution
2.18 Mineral Oil Light
2.19 Pooled Human Serum (PHS), Invitrogen™ product code 34005100
2.20 Rabbit Complement, Invitrogen™ product code 31021100
2.21 HLA Positive Control Serum, Invitrogen™ product code 1145005
3  **Sample Requirements:**

3.1 A lymphocyte suspension is prepared from 10ml whole blood collected into a heparinized, ACD, or sodium citrate vacutainer tube. Cell concentration is adjusted to 2-3 x 10^6 cells/ml. The lymphocyte suspension must be prepared within 48 hours of collection of whole blood for optimum viability. The suspension is to be stored at room temperature until use.

3.2 A viability of greater than 80% without excessive contamination of non-lymphocyte cells is essential for optimum performance of HLA antisera.

3.3 Granulocyte contamination or a high background of non-viable lymphocytes may cause a false positive reaction.

3.4 Platelet contamination may result in incomplete lysing of lymphocytes causing a false negative reaction.

3.5 Lymphocytes may be prepared according to the following procedure or additional methods which are listed in the ASHI Laboratory Procedure Manual.³

4  **Lymphocyte Suspension Preparation:**

4.1 Collect 10 ml of whole blood into a heparinized, ACD, or sodium citrate vacutainer tube. (EDTA has been found to be an unacceptable anticoagulant.³) Store the sample at room temperature until use.

4.2 Centrifuge the blood for 10 minutes at 700-900 x g to obtain buffy coat.

4.2.1 This can also be done by use of an aggregating agent such as 5% Dextran as follows:

4.2.1.1 Mix 2 ml of 5% Dextran with 10 ml of whole blood and allow red cells to sediment at 37°C for 15 minutes.

4.3 Using a Pasteur pipette, carefully remove the buffy coat (approximately 2 ml) and transfer to a clean 17x100 mm tube containing 5 ml of HBSS. Mix well.

4.4 Dispense 4 ml of Ficoll-Hypaque (FH) gradient solution (22°C) into a clean 17x100 mm tube. Carefully layer the buffy coat suspension over the FH gradient solution.

4.5 Centrifuge for 20 minutes at 700 x g. After centrifugation the mononuclear cells can be found as a narrow band at the interface between the plasma/diluent and gradient solution.

4.6 Aspirate all of the mononuclear cell layer and transfer to a 17x100 mm tube. Dilute with 4 ml of HBSS.

4.7 Centrifuge for 10 minutes at 600 x g. Remove supernatant, gently resuspend cell pellet, add 4 ml HBSS, and centrifuge for 10 minutes at 600 x g.

4.8 Remove supernatant and resuspend cell pellet in 1 ml of RPMI-1640 with 20% PHS.

4.9 Examine the cell suspension on a hemacytometer. Assess purity and perform a cell count. Adjust the cell concentration to 2-3 x 10^6 cells/ml.

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³ For In Vitro Diagnostic Use
4.10 Perform a viability test as follows.

4.10.1 Add one drop of Trypan Blue and one drop of cell suspension in a clean tube, then mix well.

4.10.2 Incubate the mixture at room temperature for 15 min.

4.10.3 Examine the cell viability on a hemacytometer. Viable cells have intact cell membranes and thus appear smooth; they are able to exclude the Trypan blue and therefore will be colorless. Non-viable cells do not have intact cell membranes and thus do not appear smooth; they are unable to exclude the Trypan blue and therefore will be colored.

4.10.4 The lymphocyte suspensions are ready for use in the Class I Microlymphocytotoxicity Test using the dye exclusion method.

5  B and T Cell Separation:

5.1 Nylon Wool Procedure:

B and T lymphocytes are easily separated by use of the nylon wool technique. B cells and macrophages have the property of adhering to nylon wool, whereas T cells do not. Refer to the 4th Edition ASHI Laboratory Manual; Section 1.A.6 “Nylon Wool Separation of T and B Lymphocytes” for B and T cell separation procedure.

5.2 Bead Procedure:

5.2.1 Magnetic beads are available from several manufacturers.

5.2.2 HLA Cell Prep I and HLA Cell Prep II (Invitrogen™) have been tested for use with all Class I & II antisera. Refer to manufacturers Instructions for Use.

5.2.3 Bead purified B cells and T cells are generally prestained using CFDA (Carboxyfluorescein Diacetate) or Ethidium Bromide.

6  Microlymphocytotoxicity Test:

6.1 If Freeze-Dried sera are used, reconstitute sera as follows.

6.1.1 Reconstitute the serum with a 1 ml of sterile distilled water.

**NOTE: Accurate measurement of the distilled water is critical.**

6.1.2 Dispense reconstituted antisera directly onto test trays (see Section 6.4) or into smaller units for future use.

6.1.3 Aliquots can be frozen and should be stored at or below -55°C. Reconfirm reactivity prior to use.

6.2 If Frozen sera are used, thaw the sera as follows.

6.2.1 Thaw a vial of serum in 37°C water bath and remove immediately upon total thawing.
For In Vitro Diagnostic Use

6.2.2 Dispense thawed antisera directly onto test trays (see Section 6.4) or into smaller units for future use.

6.2.3 Aliquots can be frozen and should be stored at or below -55°C. Reconfirm reactivity prior to use.

6.3 Prepare a lymphocyte suspension of at least 80% viability without excessive contamination of non-lymphocyte cells.

6.4 Using a pasteur pipette or automatic tray oiler, add mineral oil to each well on the test trays.

6.5 Using a 50 µl syringe, add 1 µl of appropriate Invitrogen™ HLA antisera to the test well, below the mineral oil.

6.6 Using a 50 µl syringe, add 1 µl of lymphocyte suspension (approximately 3,000 lymphocytes) to the top each test well, being careful not to touch the antisera. Examine each well to ensure the lymphocyte suspension and antiserum have mixed.

6.7 Incubate the microtrays at room temperature (22°C ± 3°C).

   Class I (dye exclusion) 30 min
   Class I (fluorescence) 30 min
   Class II (fluorescence) 45 min

6.8 Using a 50 µl or 250 µl syringe, add 5 µl of rabbit complement to the test wells, being careful not to touch the antisera/lymphocyte mixture with the syringe tips.

6.9 Incubate the trays at room temperature (22°C ± 3°C).

   Class I (dye exclusion) 60 min
   Class I (fluorescence) 50 min
   Class II (fluorescence) 60 min

6.10 Using a 100 µl syringe or equivalent, add 2 µl of filtered 5% aqueous Eosin Y to each test well and incubate at room temperature (22°C ± 3°C) for 3-5 minutes. Be careful not to touch the antisera/lymphocyte mixture with the syringe tips. Omit this step if you are using a Fluorescein based assay.

6.11 Using a 250 µl syringe, add 5 µl of filtered neutralized formalin to each test well, being careful not to touch the antisera/lymphocyte mixture. Omit this step in the Fluorescein based assays.

   6.11.1 Fluorescein based assays: Add 5 µl of propidium iodide in quenching solution to each of the test wells, being careful not to touch the antisera/lymphocyte mixture.

6.12 Place a cover slide over the tray and let plates stand at room temperature for 15 minutes to allow the lymphocytes to settle.

   6.12.1 Trays stained with Eosin Y can be read after one hour or the following day.

   6.12.2 Trays stained with Fluorescein can be read after 30 minutes or the following day.
Note: Reading a fluorescein-stained tray after 36 hours may result in increased false positives.

6.13 Observe the test microscopically at 150 x magnification under phase contrast illumination.

7 Interpretation:

7.1 Dead cells (those possessing the antigen) absorb the dye, appear enlarged and darkened and show distinct nuclear detail. Viable cells (those lacking the antigen), exclude the dye, appear slightly brighter and smaller in size as compared to dead cells.

7.2 Alternatively, fluorescently labeled viable cells are green and nonviable cells are red.

7.3 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>Unreadable</td>
</tr>
</tbody>
</table>

8 Performance Standards:

8.1 Specificity and Sensitivity

8.1.1 Invitrogen™ HLA Antisera have been extensively tested by internal and external screening against a well qualified lymphocyte panel containing various ethnic groups. The dilutions and specificity of each Invitrogen™ HLA Antisera have been determined by titration (using serial dilutions) against lymphocyte suspensions with known HLA types. Each serum is used at its optimal dilution to achieve the highest reaction value while maintaining specificity. Most sera have R values of 0.8 or better. Information contained in the Certificate of Analysis provided for each lot of trays will assist in explaining unexpected reactions.

8.1.2 Invitrogen™ HLA Positive Control Serum (rabbit anti-human lymphocyte serum) should be included on each test tray.

8.1.3 Invitrogen™ Pooled Human Serum (PHS) should be used as Negative Control Serum on each test tray. This serum has been screened and found to be negative for lymphocytotoxic antibodies against T cells and B cells by the standard microlymphocytotoxic assays.

9 Troubleshooting:

9.1 High background or false positives may be attributed to:
9.1.1 Sample has low cell viability or damaged cells. Remove dead cells before adding to the typing trays, or prepare another cell suspension.

9.1.2 Sample is contaminated with granulocyte or other non-lymphocytes. Remove non-lymphocyte cells before adding to the trays, or prepare another cell suspension.

9.1.3 Reagents containing toxic substances or that have pH outside the normal physiologic range may cause cell damage. Be sure reagents used have been properly tested.

9.1.4 Complement is too strong or has high toxicity level.

9.1.5 Incubation time is too long. Be sure to follow this Instruction for Use closely for incubation time based on cell isolation techniques.

9.1.6 Incomplete reaction or false positives can be caused by cell or serum carry-over.

9.2 Weak reaction or false negatives may be attributed to:

9.2.1 Sample is too concentrated. Be sure the cell concentration is adjusted to 2-3 x 10^6 cells/ml.

9.2.2 Sample is contaminated with platelets.

9.2.3 Sera or reagents have pH changed due to exposure to CO₂ or bacterial contamination. Use fresh reagents and check pH before use.

9.2.4 Complement is too weak or inactivated prior to addition to the trays.

9.2.5 Incubation temperature is improper. Low temperatures cause slower reaction, whereas high temperatures cause degradation of thermolabile components. Be sure the incubation temperature is 22°C ± 3°C.

9.2.6 Incubation time is too short. Be sure to follow this Instruction for Use closely for incubation time based on cell isolation techniques.

10 Limitations and Precautions:

10.1 Rabbit complement is a critical reagent in the lymphocytotoxicity test procedure and can vary from lot to lot. Unsatisfactory complement will result in weak or false negative reactions or high background in the negative control. The activity of rabbit complement can be determined by testing with appropriate positive and negative controls as well as with known lymphocytes and antisera.

10.2 Invitrogen™ HLA-ABC and DR Complement have been individually tested to meet minimum titers and 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 an existing or known lot. A suitable rabbit complement should exhibit a strength reading of 8 with the positive control and most of the
positive reactions. It should exhibit a strength reading of 1 with the negative control and most of the negative wells.

10.3 HLA testing using the Invitrogen™ HLA Antisera must be performed in the presence of a qualified Director, Technical Supervisor and/or General Supervisor following accepted laboratory standards. We must emphasize that these products are for professional use only.

10.4 Samples should be stored at room temperature until use and EDTA should not be used as an anticoagulant for sample collection.

References


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