Chapter 3: Flow Cytometry/Luminex

Section: Application Modules

Module: (b) HLA-B27 Typing – Flow

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Objectives

- Understand the clinical rationale for determining if an individual is HLA-B27 positive
- Understand the general testing process and requirements for HLA-B27 typing by flow cytometry
- Understand the pros and cons of HLA-B27 screening by flow cytometry
- Understand the ASHI Standards that are applicable to single antigen typing by flow cytometry
Introduction

The association between HLA-B27 and ankylosing spondylitis, a chronic inflammatory disease of the axial musculoskeletal system, has been demonstrated for over 30 years. The HLA-B27 antigen has also been linked to other rheumatic disorders including Reiter’s disease, acute anterior uveitis and inflammatory bowel disease. HLA-B27 testing is routinely performed to screen for ankylosing spondylitis as 90% of patients with the disease are HLA-B27 positive as compared to 8% of healthy individuals.

HLA-B27 testing can be performed by several methods including complement-dependent cytotoxicity (CDC), DNA-based typing and flow cytometry. Testing by CDC or DNA-based methodology typically requires specialized staffing and extensive quality control procedures that many diagnostic laboratories may not possess. Therefore, HLA-B27 screening by flow cytometry has become widely used. This type of testing may be incorporated into laboratories already performing immunophenotyping or other flow cytometric assays, rather than specialized HLA laboratories. HLA-B27 screening by flow cytometry can be an inexpensive means of typing, particularly for large test batches.

The reagents used in HLA-B27 screening by flow cytometry include a monoclonal antibody directed against HLA-B27 antigen, conjugated with fluorescein isothiocyanate (FITC), a fluorescent dye. Some testing systems also use another fluorescently labeled monoclonal antibody specific to T-lymphocytes, such as CD3. Another type of test system includes a second anti-HLA monoclonal antibody directed against a cross-reactive antigen such as HLA-B7, conjugated to a different fluorochrome. This can be used to identify and rule out cells with a cross-reactive HLA antigen. The monoclonal antibodies for the specific test system are added to the patient’s peripheral blood sample, washed several times to remove excess antibody, and run on the flow cytometer. For some test methods, an isotype control is also used to account for any non-specific binding.

To analyze the assay on the flow cytometer, a lymphocyte or T-lymphocyte population is identified. This can be done using the intrinsic (size and granularity/complexity) or extrinsic (CD3 binding) properties of lymphocytes. From the lymphocyte population, the binding of the anti-HLA-B27 antibody is determined by comparison to known negative and positive controls. This comparison is typically made using a histogram and comparing the mean or median channel shift of the control and patient samples. Most laboratories determine a fluorescence range that is acceptable for calling a specimen HLA-B27 negative or positive.
The primary disadvantage to HLA-B27 screening by flow cytometry is the cross reactivity of the anti-HLA-B27 antibody. The most common cross reactivity is found with the HLA-B7 cross reactive epitope group (CREG). Members of the HLA-B7 CREG that can be cross-reactive include HLA-B7, B13, B22, B37 and B42. If a test kit includes an anti-HLA B7 antibody in addition to the anti-HLA-B27, some cross-reactive binding can be ruled out. However, a second method of HLA typing must be used for confirmation of the presence/absence of HLA-B27 when results are ambiguous.
Methodologies

Commercial kits and reagents are available from multiple vendors. Validation of all kits and/or reagents is required prior to clinical use. The following is a summary of reagents and vendors available for HLA-B27 screening.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>Anti-HLA-B27 FITC</td>
<td>Identify cells with HLA-B27 antigen</td>
</tr>
<tr>
<td>Isotype Control (optional)</td>
<td>Negative control reagent to identify non-specific binding</td>
</tr>
<tr>
<td>Anti-CD3 phycoerythrin (PE) (optional)</td>
<td>Identify T-lymphocytes</td>
</tr>
<tr>
<td>Anti-HLA-B7 PE (optional)</td>
<td>Identify and rule out cells with HLA-B7 antigen</td>
</tr>
<tr>
<td>Whole blood lysing system</td>
<td>Remove red blood cells prior to acquisition</td>
</tr>
<tr>
<td>Phosphate-buffered saline with formaldehyde or sodium azide</td>
<td>Fixing solution</td>
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</table>

Equipment:
Flow cytometer – minimum of 2 color detection
Centrifuge
Vortex

QA/QC Information:
Optical standards for flow cytometry
Known HLA-B27 Negative and HLA-B27 Positive controls are required for each test batch

Vendor Products Available:

- Becton Dickinson (BD) HLA-B27 System
  - Components
    - Anti-HLA-B27 FITC / CD3 PE reagent
    - BD-FACS lysing solution
    - HLA-B27 Calibration Beads
  - Pros – The BD HLA-B27 System comes as a kit complete with calibration beads and analysis software. Software automatically calculates quality control criteria including minimum T-lymphocyte events and adequate separation between negative and positive populations. The user is flagged if criteria are not met.
  - Cons – Only available for BD flow cytometers. Additional costs required to purchase software.

- Stand-alone reagent tests:
- One Lambda
  - HLA-B27 FITC (clone FD705)
  - Mouse IgG2b FITC isotype control
- Beckman Coulter
  - IOTest HLA-B27 FITC (clone ABC-m3) plus HLA-B7 PE (clone BB7.1)
  - IgG2a FITC – IgG1 PE isotype control
- AbD Serotec
  - Mouse Anti-Human HLA-B27:FITC (clone HLA-ABC-m3)
  - Mouse IgG2a Negative Control:FITC
- Pros – Can be used with any flow cytometer. No specialized analysis software to purchase.
- Cons – No built in quality control features.
Protocols

Procedure: HLA-B27 Typing by Flow Cytometry

Purpose: To describe the steps involved in identifying the presence/absence of HLA-B27 by flow cytometry. HLA-B27 is strongly linked to ankylosing spondylitis and is a useful diagnostic tool for clinicians. The procedure described is intended to be an overview of the process. For specific volumes and incubation times, refer to the specific manufacturer’s instructions.

Materials:
Flow Cytometer
Centrifuge
Vortex
Micropipettes and tips
12 x 75 mm test tubes
Anti-HLA-B27 FITC
Isotype control (optional)
Anti-CD3 PE (optional)
Whole blood lysing solution
Fixing solution (PBS with 0.5% formaldehyde)

Procedure:

Staining Cells
1. Add the anti-HLA-B27 antibody (and other antibodies, as appropriate) to well mixed peripheral blood or isolated peripheral blood lymphocytes as designated by the manufacturer's instructions.
2. If using an isotype control, repeat step 1, replacing the anti-HLA-B27 antibody with the isotype control.
3. Vortex tube(s) and incubate in the dark at room temperature for 10 – 30 minutes.
4. If whole blood was used, add lysing solution to tube(s). Vortex and incubate in the dark at room temperature for 5-10 minutes.
5. To wash tubes (optional), centrifuge to pellet cells, discard supernatant. Repeat if desired.
6. Re-suspend in fixing solution.

Sample Analysis on Flow Cytometer
1. Run optical standards appropriate for the instrument and reagents being used.
2. For automated analysis (using BD HLA-B27 System), follow the prompts on the software provided by the manufacturer. Following analysis, the software will provide reports and automated analysis of the samples.

3. For manual analysis, load and run the first sample on the flow cytometer.

4. Set a gate to encompass the lymphocyte population. This can be done on a FSC vs. SSC plot or using lymphocyte specific antibodies included in staining. Allow at least 2500 events to acquire.

5. Using the gated lymphocyte population, determine the fluorescence intensity of the FITC conjugated to the anti-HLA-B27. Compare the mean channel value to the negative control to determine if the HLA-B27 phenotype is positive or negative.

6. Repeat the acquisition for the isotype control (optional). Determine if any non-specific binding confounds the results of the anti-HLA-B27 stained tube.

7. Each laboratory should establish a range for HLA-B27 positive and negative samples based on testing at least 5-10 known samples. Verification of the range should be done for each new lot of reagent.

8. If the mean channel for a sample falls outside of the positive and negative ranges established, a secondary testing method should be used for verification.

**Limitations:**

1. Validation studies must be run to determine what level fluorescence or mean channel shifts will be considered HLA-B27 positive or negative. This can be done using a panel of known positive and negative samples.

2. The BD HLA-B27 System includes additional internal quality control processes. If these fail, the run may need to be repeated. The report will indicate what quality control parameter has been violated.

3. There are a number of HLA antigens that are cross reactive with HLA-B27 and may yield indeterminate results. These samples may need to be tested using another method.

**Precautions:**

Any human blood specimen should be considered as potentially infectious and handled with universal precautions. Fixing solutions may contain sodium azide or formaldehyde. Both reagents can be harmful if swallowed or contacted with the skin. In case of contact, flush immediately with water. Sodium azide can form explosive metal azides when it comes in contact with lead and copper plumbing. If the reagent is flushed down a sink, it must be flushed with copious amounts of water to prevent azide build up.
Quality Control/Assurance

Procedure: Quality Control for HLA-B27 Typing by Flow Cytometry

Purpose: To describe the appropriate quality control measures that must be in place to perform HLA-B27 typing by flow cytometry.

Materials:
Micropipettes
Refrigerator (0-10°C)
Flow Cytometer

Standards:
1. Verify that all micropipettes used have been calibrated. Calibration is performed twice a year.
2. Ensure that the refrigerator is holding all monoclonal antibodies and lysing reagents within their appropriate temperature range for storage. The refrigerator temperature should be recorded daily.
3. Optical standards must be run and pass every time the flow cytometer is turned on for testing. The values must be stored in such a way that trends can be detected and evaluated.
4. All reagents should be used prior to the manufacturer’s expiration dates. Reagents should also be visually inspected for contamination and discarded if any contamination is detected.
5. Validation studies must be run to determine what level fluorescence or mean channel shifts will be considered HLA-B27 positive or negative. This can be done using a panel of known positive and negative samples, as determined by other methods. The ranges determined in the initial validation must be verified with each new lot of reagents used for clinical testing.
6. Positive and negative controls must be run with each assay. If controls values do not fall within acceptable ranges, the entire run must be repeated.

Corrective Action:
Any equipment or reagent that falls outside of normal testing parameters must be resolved immediately and before clinical results can be reported. In cases where positive and negative controls do not fall within the established range on multiple, subsequent runs, a secondary method of testing should be used.
Frequently Asked Questions

Question: We do all our HLA typing for transplant patients by CDC or DNA-based methods. Why would we want to consider HLA-B27 typing by flow cytometry?
Answer: Since your laboratory already has the staffing and equipment necessary for HLA typing by CDC and DNA methods, it may not be necessary to add flow cytometry as a typing method. However, HLA-B27 typing by flow cytometry can be a quick, easy and cost effective means of screening large batches of samples.

Question: There are samples that give a mean channel value that is between the negative and positive ranges. How is this possible?
Answer: There are a number of HLA antigens that are cross reactive with HLA-B27, particularly HLA-B7. The anti-HLA-B27 monoclonal antibody is likely recognizing a shared epitope in a cross reactive antigen. This would result in an indeterminate result and will require confirmatory testing using another test method.

Question: There was a problem acquiring the negative control sample. The positive control acquired properly and fell in the established range. Do I still need to repeat the test?
Answer: Yes, if either control fails, whatever the reason, the entire run must be repeated.

Question: In what cases can we not include an isotype control and still comply with ASHI standards?
Answer: ASHI standards state that for assays where direct labeling is involved, an isotype control must be used unless 3- or 4- color fluorescence staining is used for CD4 cell counting.
Standards

D.5.2.13 Laboratories performing immunophenotyping and/or single antigen typing by flow cytometry must:

D.5.2.13.1 Use specificity controls consisting of appropriate cell types known to be positive for selected standard antibodies for each lot or shipment, where applicable.

D.5.2.13.2 Use a negative control(s) reagent for each test population. It is recommended that this control consist of monoclonal antibody(ies) of the same species and subclass and be prepared/purified in the same way as the monoclonal(s).

D.5.2.13.3 Where indirect labeling is involved, use a negative control reagent that is an irrelevant, isotype-matched primary antibody and the same secondary antibody(ies) conjugated with the same fluorochrome(s) used in all relevant test combinations.

D.5.2.13.4 Where direct labeling is involved, use a negative control reagent that is an irrelevant antibody conjugated with the same fluorochrome and at the same fluorochrome:protein ratio used in all relevant test combinations unless 3- or 4-color fluorescence staining is used for CD4 cell counting.

D.5.2.13.5 Employ gating strategies to assure that the population of interest is being selected without significant contamination.

D.5.2.13.6 Ensure the appropriate definition and purity of cell populations by the use of either a multi-color technique or other documented method.

D.5.2.13.7 Base conclusions about abnormal proportions or abnormal numbers of cells bearing particular internal or cell surface markers using comparison with local ‘control’ data obtained with the same instrument, reagents and techniques.
Literature Cited and Recommended Reading


IOTest HLA-B27-FITC/HLA-B7-PE Product Insert. Beckman Coulter 2008
