

HLA B27 TYPING

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1. INTRODUCTION

1.1. HLA-B27 is a Class1 histocompatibility antigen found on surface of all nucleated human cells. A strong association of HLA-B27+ individuals with ankylosing spondylitis (AS), a chronic inflammatory disease affecting the axial musculo-skeletal system, has been demonstrated in greater than 90% of patients with AS. It is evident that this antigen is common to patients with Reiter's syndrome, anterior acute uveitis, psoriatic arthritis and inflammatory bowel disease (13). Thus screening for HLA-B27 is useful and is performed by many diagnostic laboratories.

1.2. Traditionally the lymphocytotoxicity assay was used to determine HLA status (1) though time consuming and expensive to perform. Alternate methods that have been developed to speed up HLA-B27 screening have included: flow cytometry, DNA-based typing using polymerase chain reaction (PCR) assays and enzyme-linked immunosorbent assays (15)

1.3. The advent of monoclonal antibodies (mAb) to the HLA-B27 antigen led the way for a flow cytometric method (2). However, HLA-B27 screening is hampered by that the fact it is a member of the large HLA-B7 cross-reacting group (CREG) of antigens which shares common epitopes (15). Hence when individuals with homozygous B7 expression are screened, a false positive result will be likely as this allelic configuration has a population frequency of approximately 6% compared to 9% with HLA-B27 in the Caucasian population (14).

1.4. The use of flow cytometry for screening is also hampered by the specificity and sensitivity of commercial clones available on the market for HLA-B27. All three of the common clones, ABC-m3, FD705 and GS145.2, have shown to cross react with other HLA-B antigens and some showed a lack of sensitivity in recognizing all subgroups of HLA-B27 antigens. To combat these issues of specificity and sensitivity, a number of studies have recommended that two different anti-HLA-B27 clones be used for more accuracy and reliability in flow cytometry screening (14-15).

2. SPECIMEN COLLECTION, TRANSPORT and INTEGRITY

2.1. Specimen Collection

2.1.1. Universal precautions should be strictly observed when collecting blood samples (see AFCG guidelines on Laboratory Safety).

2.1.2. Heparin, EDTA or ACD (solution A) anticoagulated specimens may be processed immediately or up to 48 hours after collection. A FBC and differential result may be useful and should accompany sample where possible.

2.1.3. Specimens more than 48 hours old or unlabelled or incorrectly labelled or of insufficient volume should be recollected.

2.1.4. Frozen cells (see Appendix 1) or gradient separated peripheral blood mononuclear cells (PBMC) may be used to monitor for immunofluorescence variability.

2.2. Specimen Transport

2.2.1. Packaging, labelling and transport of specimens should comply with all current local, state, national and international regulations for the regions through which the specimens will be transported.

2.2.2. Specimens should be maintained at 16^o-22^o C in a light and leak proof container. Temperatures below 10^oC or above 37^oC must be avoided.

2.3. Specimen Integrity

2.3.1. Visually inspect the specimen for clots, haemolysis or container defects. Where possible, recollect the sample if the specimen shows any visual signs of deterioration.

2.3.2. Specimens that are collected or transported outside of these guidelines if tested, then result should be interpreted with caution and the report should indicate the deficiency under which testing was performed.

3. SPECIMEN PROCESSING

3.1. Whole blood lysis of red cells is recommended for routine analysis. Most methods of HLA-B27 analysis (3-9) employ either a single colour direct fluorescence for HLA-B27, with or without an unlabelled anti-B7 monoclonal (4,15), or in a dual colour assay in combination with anti-CD3 or HLA-B7 antibody. Gating on the lymphocyte population or CD3+ cells is usual.

3.2. Several erythrocyte lysing reagents are available and the laboratory should choose a method that provides optimal lysing in every assay. When using commercial reagents, manufacturer's recommended protocol should always be followed unless data are available confirming that any modifications do not adversely affect results. Valid results can also be obtained from gradient density separated PBMC. These cells can be stored in frozen aliquots and used as quality control material (see Appendix 1: Cell Freezing and Thawing).

3.3. Where possible a full blood count and differential can be performed before processing. If this is not possible, each laboratory must have a procedure to identify specimens with abnormal WBC counts and correct for any associated artifacts. Specimens with pronounced leucopenia may have insufficient cells for flow cytometric analysis, thus requiring a larger volume of sample or a buffy coat preparation. Conversely, samples with leucocytosis may require less volume for testing to ensure antigen excess due to increased cell count would not lead to possible false negative results.

3.4. The laboratory should validate the amount of antibody which will resolve negative staining cells from positive staining cells and provide the optimal staining intensity for positive cells based on the laboratory's method of sample preparation and mAb clones used. The use of isotype controls and control cells with known HLA types: HLA-B7 neg, HLA-B27 neg, HLA-B27 pos and HLA-B7 pos would be useful for establishing method. These samples can be obtained from tissue typing reference centers and stored for latter use.

3.5. The assay performance should then be checked for every new batch of antibody and after every instrument service using known negative and positive controls where possible. To check for drifts in the PMT detector used for fluorescent detection, calibration fluorescent beads can be utilised.

4. SAMPLE ANALYSIS

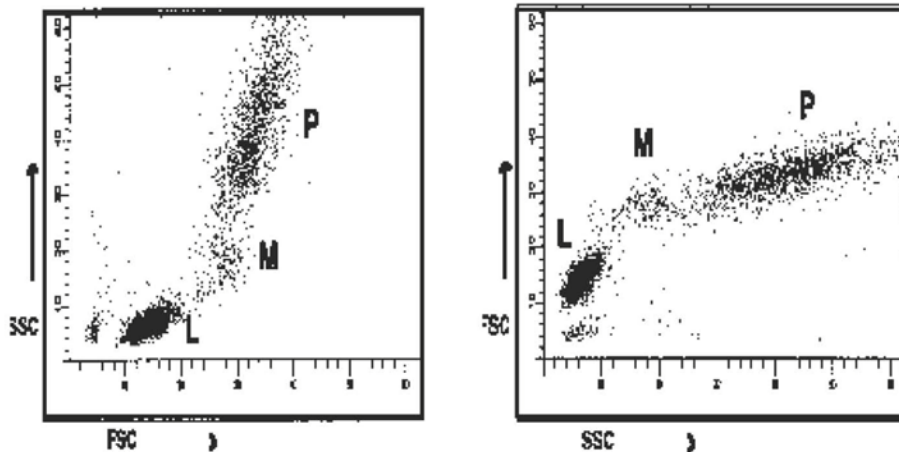
4.1. Test order for acquisition- set gate on the first tube and ensure that the cells of interest are not excluded and minimise contamination within gate by setting the discriminator/threshold to exclude platelets, debris etc. All control specimens used for validation of assay should be run first before patient samples. If an isotype control is used it should be run before the test sample.

4.2. Assessment of specimen viability may be desirable for old collections. Due to biohazard concerns, it is recommended that all samples be appropriately fixed prior to analysis on the flow

cytometer. To distinguish cells which were non-viable after fixation, ethidium monoazide (EMA) can be utilised (12).

4.3. Figure 1a Figure 1b.

4.4. The definition of a lymphocyte gate is shown in Figures 1a and 1b.



Figures 1a and 1b. Representation of common ways of displaying FSS vs SSC from whole blood lysed preparations. (L = predominantly lymphocytes, M = predominantly monocytes, P = predominantly polymorphonuclear leucocytes).

4.4. To ensure reasonable statistical confidence- count at least 2000 gated events in each sample will provides a 95% confidence interval. However, the counting of 2000 gated events may not be achievable in severely leucopenic samples.

4.5. Each laboratory should establish acceptable limits for cell contaminating and debris and determine when corrective actions are required. Satisfactory values for lymphocytes are seeing 90% of all lymphocytes. If levels of contamination by non lymphoid cells cannot be minimised to within acceptable limits even after reprocessing, then test results should be questioned and recollection should be requested.

5. DATA REPORTING

5.1. Report all unique patient identifiers.

5.2. Report result as: positive, negative or indeterminate result. Indeterminate results should have instructions for referral for HLA typing by lymphocytotoxicity or PCR analysis.

5.3. Report description and results of confirmatory testing where applicable.

6. QUALITY ASSURANCE

6.1. Each laboratory should determine mean channel of fluorescence cut-offs at which HLA-B27 is excluded and demonstrated. The nature of the assay is such that in some samples the mean channel of fluorescence falls within these cut-offs ('grey area'), these samples would need for

further testing. To avoid false positive assignment due to cross-reactivity of monoclonal antibodies, HLA-B27 screening should require the use of two different mAb clones (14-15).

6.2. The laboratory should belong to and participate in a recognised external quality assurance program as provided by RCPA Quality Assurance (program HLA B27).

6.3. The laboratory should determine the level of test variability by preparing and analysing a positive sample at least six times over a period of 48 hrs after venipuncture.

6.4. If possible set up an HLA-B27 positive sample once a week or after post servicing of instrument, especially when setting up this assay. Controls cells frozen from typed donors, stabilised whole blood controls or cell line available from commercial manufacturers may be used. Samples demonstrating HLA-B27 status from the previous day's run may also be used as control material.

7. REFERENCES

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8. APPENDIX 1: CELL FREEZING AND THAWING

8.1. Reagents

8.1.1. Blood cell source. These may be density gradient separated peripheral blood mononuclear cells (PBMC), PBMC from buffy coats or transformed B cell lines. This method may not be suitable for whole blood techniques.

8.1.2. Cell culture media -20-50% foetal calf serum in RPMI or other balanced salt solution. This should be cooled for freezing, warmed for thawing.

8.1.3. Freeze mix -dimethyl sulphoxide (DMSO) at 20% in cell culture media.

8.1.4. Cryotubes -prelabelled and chilled if possible.

8.2. Procedure

NOTE: All preparation should be carried out on ice as DMSO is toxic to cells at room temperature.

8.2.1. Count PBMC.

8.2.2. Gently centrifuge cells and resuspend in cell culture media at 107 per ml.

8.2.3. Place cells in a small beaker on ice and drop wise add to the centrifuge tube an equivalent volume of pre-cooled freeze mix, mixing well.

8.2.4. Pipette into 1mL aliquots into cryotubes.

8.2.5. As quickly as possible place tubes in an insulated container into -70°C overnight. Cells may be stored at this temperature for many months if liquid nitrogen storage is unavailable.

8.2.6. Cells should be thawed as quickly as possible by warming the cryotube in a 37°C water bath.

8.2.7. Pipette into a centrifuge tube containing pre-warmed cell culture media or equivalent and centrifuge as before.

8.2.8. Reconstitute cell pellet as desired.