HLA-B27 Typing: Evaluation of an Allele-Specific PCR Melting Assay and Two Flow Cytometric Antigen Assays

Michael T. Seipp,1* Maria Erali,1 Rae Lynn Wies,2 and Carl Wittwer1,2,3

1ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, Utah
2ARUP Laboratories, Salt Lake City, Utah
3Department of Pathology, University of Utah Medical School, Salt Lake City, Utah

Background: Human leukocyte antigen B27 (HLA-B27) is a major histocompatibility complex class 1 molecule that is strongly associated with the disease ankylosing spondylitis. Testing for HLA-B27 is of diagnostic value because 90% of patients with ankylosing spondylitis have the B27 antigen. Two commonly used HLA-B27 flow cytometric assays are commercially available.

Methods: An allele-specific polymerase chain reaction (PCR) melting assay for HLA-B27 was compared with two available antigen assays on 371 clinical samples. The accuracy of the assays was measured by receiver operating characteristic analysis using the PCR method and sequencing as the reference standard.

Results: When PCR results were compared with those of the antigen assays, complete concordance was observed except for five discrepant results that were resolved by sequence analysis. Using DNA sequencing as the gold standard, the sensitivity and specificity of PCR were 99.6 and 100.0, those of the best single antigen assay were 98.2 and 97.6, and those of a reflex combination of both antigen assays were 98.8 and 97.6.

Conclusions: The allele-specific PCR melting assay for HLA-B27 genotyping is easy to perform and has better sensitivity and specificity than antigen assays. The performance of the two flow cytometric antigen assays depends on the antibody used and the positive cutoff values assigned. © 2004 Wiley-Liss, Inc.

Key terms: HLA-B27; allele-specific PCR; melt analysis

Human leukocyte antigens (HLAs) are products of genes found at the human major histocompatibility complex. These antigens are important for the immune system to be able to differentiate “self” from “non-self” and are essential for communication between cells of the immune system. Although HLA molecules are best known for their role in transplantation, certain HLA molecules are associated with specific diseases. HLA-B27 is strongly associated with a number of rheumatic diseases, including ankylosing spondylitis, Reiter’s syndrome, acute anterior uveitis, and inflammatory bowel disease (1). Ankylosing spondylitis (AS) is an inflammatory systemic disease that affects the spine and the sacroiliacal and peripheral joints. The heart, bowels, and skin may also be involved in AS. HLA-B27 testing is of diagnostic value because 90% of patients who have AS have the B27 surface antigen compared with only 8% of healthy individuals. In addition, the risk of disease increases threefold if the HLA-B27-positive person has a first-degree relative with AS. AS affects men and women, although the disease has an overall male predominance. Spondyloarthropathies usually begin in the late teens and early 20s but may also present earlier in childhood or at an older age (2).

Currently, most HLA-B27 testing is performed with surface antigen tests. These tests are relatively inexpensive and easy to use but require viable cells for analysis. Two commercially available antibodies are commonly used. These antibodies may cross-react with other HLA-B surface antigens, especially B7 and B40 (3). The cross-reactivity of these antibodies can compromise the accuracy of the results generated in the antigen assays. We used a polymerase chain reaction (PCR) melting assay specific for the HLA-B27 allele as a reference standard to evaluate the accuracy of two commercial HLA-B27 assays and resolve discrepant results by sequencing.

*Correspondence to: Michael T. Seipp, ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108. E-mail: seippmt@aruplab.com
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MATERIALS AND METHODS

Samples

Whole blood samples were acquired in K₃ ethylene-diaminetetra-acetic acid, Na-heparin, and citrate phosphate-dextrose tubes and analyzed by flow cytometry. All samples were submitted to our institution for clinical evaluation by HLA-B27 antigen testing. Samples for the study were selected to include one-third clearly positive, one-third clearly negative, and one-third near to the cutoff of one of the flow cytometric assays (GS145.2 antibody, see below) and were de-identified as required by the Health Insurance Portability and Accountability Act of 1996 regulations. This selection process enriched for samples near the cutoff (from ~10% of samples normally received to ~33%).

DNA from whole blood stored at 4°C was extracted with the Qiagen QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) or the Roche MagNA Pure LC System (Roche, Indianapolis, IN, USA) according to the manufacturers’ instructions and analyzed by the allele-specific HLA-B27 PCR assay. The final DNA concentration for both extraction methods was approximately 40 ng/μl and was quantified by absorbance at A₂₆₀ for dilution experiments.

GS145.2 Antibody Assay

Samples were tested for the presence of the HLA-B27 surface antigen with the GS145.2 antibody (HLA-B27 Kit, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) according to the manufacturer’s instructions. Samples were stained with anti–HLA-B₂⁷*fluorescein isothiocyanate/CD3*phycoerythrin monoclonal antibody reagent, followed by lysis of red blood cells and fixation of white blood cells with formaldehyde. Samples were processed in a FACSCalibur instrument, calibrated with FACSComp software and analyzed with HLA-B27 software (Becton Dickinson Immunocytometry Systems).

FD705 Antibody Assay

Samples were tested for the presence of the HLA-B27 antigen with one lot of FD705 antibody (HLA-B27 Kit, One Lambda, Inc., Canoga Park, CA, USA) according to the manufacturer’s instructions. Samples were stained with anti–HLA-B₂⁷*fluorescein isothiocyanate monoclonal antibody reagent, followed by lysis of red blood cells and fixation of white blood cells with formaldehyde. Samples were processed in a FACSCalibur instrument, calibrated with FACSComp software, and analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems).

PCR Procedure

The HLA-B27 target was a unique 135-bp fragment in exon 3 of the HLA-B locus, which covers codons 91 to 136 (6,7). The forward primer, E91s, is unique to B27 alleles (7) and the reverse primer, E136as, is specific for all HLA-B
alleles. The primer sequences and the consensus sequences for the major HLA-B27 alleles are presented in Figure 1. A 268-bp fragment of the human β-globin gene was used as the target for an internal amplification control. The primers used were BG2 (forward, GAAGAGCAGAGGTAC) and BG1 (reverse, CAACTTCATCCACGTCACC) (8). All primers were prepared by Qiagen-Operon (Qiagen, Inc.).

PCR was performed in 10-μl volumes with 1× LightCycler FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN, USA), 5 μM each of the primers (E91s, E136as, BG1, and BG2), 2 mM MgCl₂ (including 1 mM MgCl₂ contributed by the LightCycler Master solution), 1 U/reaction heat-labile uracil-DNA glycosylase (Roche), and 1 μl of extracted DNA. PCR was done on a LightCycler (Roche) with an initial hold at 95°C for 10 min to allow polymerase activation and deactivation of the uracil-DNA glycosylase. The hold was followed by 35 cycles at 95°C for 0 s, 68°C for 8 s, and 72°C for 12 s. Melting curves were generated after denaturation at 95°C for 10 s, annealing at 65°C for 20 s, and heating at a ramp rate of 0.1°C/s to 95°C with continuous fluorescence acquisition. All heating and cooling steps during PCR were done with ramp rates of 20°C/s (8). Crossing points were calculated as the second derivative maximum, and derivative melting curves were generated by using default settings of the LightCycler. Unknown samples were deemed positive or negative based on a melting temperature (Tm) 2 standard deviations of the Tm of the positive or negative controls.

Sequencing

HLA-B sequence-based typing of selected samples was performed on the ABI PRISM 377 DNA Sequencer using the ABI HLA-B Amplification and Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Data Analysis

Statistical analysis for comparing the GS145.2 antibody, FD705 antibody, and the DNA assays was performed in EP Evaluator (David G. Rhoades Associates, Inc., Kennet Square, PA, USA) and Excel (Microsoft, Redmond, CA, USA). Sensitivity and specificity were calculated by using a combination of the DNA assay and sequencing as a reference standard. Receiver operating characteristic (ROC) curves are presented as sensitivity versus 1-specificity (9).

RESULTS

Allele-Specific PCR Melting Assay for HLA-B27

Melting curves generated by plotting fluorescence versus temperature and the corresponding derivative plots are presented in Figure 2. Amplification of the HLA-B27 allele generated products with Tm values of 88.2 ± 0.4 with a range of 87.8 to 88.6 for Qiagen-extracted samples (n = 162) and 87.9 ± 0.6 with a range of 87.3 to 88.5 for MagNA Pure-extracted samples (n = 53). In samples that were negative for the HLA-B27 allele, the β-globin gene was amplified and the products had Tm values of 85.4 ± 0.4 with a range of 85.0 to 85.8 for Qiagen-extracted samples (n = 111) and 85.0 ± 0.4 with a range of 84.6 to 85.4 for MagNA Pure-extracted samples (n = 45). The difference in Tm between the two extraction procedures was significant for positive (P < 0.0001) and negative (P = 0.0002) samples (paired t test). The sensitivity and specificity for 371 samples were 99.6% and 100%, respectively, using DNA sequencing as the gold standard when results were discrepant between the DNA and antigen assays.

A dilution series of DNA at 40, 20, 10, 5, 2.5, and 0 ng/reaction was run in triplicate in the HLA-B27 PCR assay. The curve generated from plotting the crossing point versus the log of the DNA concentration had a regression equation of y = −2.17x + 25.48 with an R² value of 0.96. Amplification was observed with starting DNA concentrations of 2.5 ng/reaction, indicating that the HLA-B27 allele can be detected at DNA concentrations at least as low as 420 copies/reaction.

Flow Cytometric GS145.2 Antibody Assay

The results for 371 samples analyzed by the GS145.2 antibody assay are compared with the DNA results in Table 1. All samples with median fluorescence intensity (MFI) greater than or equal to 165 in the GS145.2 antibody assay were positive by DNA analysis. All samples with MFI

![Fig. 2. Melting curves (A) and derivative melting curves (B) from representative HLA-B27-positive (black dashes), HLA-B27-negative (solid gray), and no template control (black line) samples. The Tm of positive samples (~89°C) results from the HLA-B27 amplicon. The Tm of negative samples (~86°C) results from the β-globin amplicon.](image-url)
less than or equal to 149 were negative. Samples with MFI values of 150 to 164 were variably positive or negative in the PCR assay and were considered as indeterminate by the GS145.2 antibody assay.

The manufacturer’s protocol for the GS145.2 antibody assay recommends that samples be considered positive when the MFI is greater than or equal to 143. Using this criterion, the flow cytometric assay had a sensitivity of 100.0% and a specificity of 71.4%. All positive results were identified with an MFI cutoff of 143, but 14.8% of the positive samples were falsely positive.

Examination of the PCR results for samples within the indeterminate range of 150 to 164 MFI (Table 1) suggests that the specificity of the GS145.2 antibody assay could be improved with only a minor loss in sensitivity if the cutoff point is placed between 154 and 155 MFI. With this cutoff, the GS145.2 antibody assay had a sensitivity of 95.2% and a specificity of 88.6%.

### Flow Cytometric FD705 Antibody Assay

The results for 205 samples analyzed by the FD705 antibody flow cytometric assay are compared with DNA results in Table 2. A cutoff for the FD705 antibody assay at a MFI greater than or equal to 124 resulted in a sensitivity of 98.2% and a specificity of 97.6%.

### Combination Flow Cytometric Assay With Two Antibodies

Using two different anti-HLA-B27 antibodies can improve overall sensitivity and specificity for HLA-B27 antigen detection (3). A combination assay was evaluated in which the GS145.2 assay was used for initial detection, with the FD705 assay as a reflex test for those samples that were in the indeterminate range of 150 to 164 MFI. The sensitivity of the combination assay was 98.8% and the specificity was 97.6%.

### ROC Comparison

A parametric ROC graph was generated by changing the sensitivity and plotting 1-specificity for the GS145.2 antigen (manufacturer’s and modified cutoffs), FD705 antigen, and the allele-specific DNA assays (9). The accuracy (sensitivity and specificity) of the allele-specific DNA melting assay was superior to that of the antigen assays (Fig. 3).

### DISCUSSION

Currently, most HLA-B27 testing is performed by surface antigen analysis using GS145.2 and FD705 clones.

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The GS145.2 antibody cross-reacts with HLA-B7 and HLA-B40 (3). This limitation is noted by the manufacturer and is the reason for many indeterminate results reported by flow cytometry.

In contrast, the primers for the DNA assay are specific for B27 and do not amplify other B alleles (7). With the DNA melting assay, some variation of Tm can occur when using different extraction methods, most likely due to the difference in ionic strength of the elution buffers. This variability requires that each laboratory establish its own reference ranges and that the DNA extraction method remain constant.

The frequency of false-positive and false-negative results with the antigen tests can be adjusted by shifting cutoff values. The GS145.2 test has an indeterminate region, where positive and negative results are obtained with the DNA assay. The manufacturer of the GS145.2 test recommends a cutoff below this indeterminate region that increases the overall sensitivity of the test but sacrifices specificity. Increasing the cutoff can increase the specificity of the test with a slight loss in sensitivity. In contrast, the FD705 test has a more distinct cutoff point as judged by the DNA assay. The GS145.2/FD705 combination antigen assay data indicate that this algorithm can be used as an alternative method for evaluating the indeterminate GS145.2 assay results. Evaluation of the GS145.2, FD705, and the GS145.2/FD705 combination antigen tests using the DNA assay and sequencing as a reference standard shows that, of the antigen assays, the FD705 antibody assay and the GS145.2/FD705 combination assay have the best sensitivity and specificity.

Discrepent samples C and D had Tm values that were not characteristic for B27-positive and B27-negative samples, respectively. The Tm values of these samples may have shifted due to polymorphisms present in the products. Any sample in the allele-specific PCR assay that generates a product with a Tm outside the expected range should be evaluated by flow cytometry and/or sequenced to verify the HLA-B27 type.

Discrepent sample E tested HLA-B27 negative by PCR but positive by the GS145.2 antibody assay. DNA sequencing showed that this sample matched the B*2707 allele (Fig. 1). When the sequence of the B*2707 allele is examined, there is a mismatch at the 3' end of the forward primer E91s that will not allow amplification of this allele (10,11). Sequencing the B*2707 subtype requires a different primer from the E91s (12). With the 3' mismatch of the forward primer, sample E had two polymorphisms under the reverse primer. Due to the primer mismatches, sample E was not detected in this PCR assay. HLA B*2707 is a rare subtype of HLA-B27. The overall allelic frequencies of HLA-B27 are 2.0 to 4.6, 0 to 2.0, and 0 to 6.0 within the white, black, and Asian populations, respectively. Because the allelic frequency of the B*2707 subtype within the HLA-B27 positive population is lower than 1% (13–17), the estimated false-negative rate of the DNA HLA-B27 assay resulting from the B*2707 subtype should be lower than 0.05%.

The labor involved took approximately 6 min for each antigen assay and 20 min for the DNA assay. Overall test costs including kit cost, disposables, and reagents were approximately $8.00, $4.00, and $20.00 for the GS145.2, FD705, and the DNA assays, respectively. These costs can be decreased by using home brew reagents.

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LITERATURE CITED


