Validation Protocol for the BD™ HLA-B27 Kit

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Introduction

Before reporting patient test results, the flow cytometry laboratory must go through a process of validation to demonstrate that it can obtain performance specifications comparable to those established by BD Biosciences for the following performance characteristics:

- Accuracy (method comparison)
- Intra-assay precision (within-run precision)
- Inter-assay precision (between-run precision)
- Interference
- Carryover studies
- Linearity
- Stability Studies

The process of validation includes the following steps:

- Review the instruction manual of the test to be implemented
- Review technical data sheets (TDSs) of tests to be implemented
- Review the standard operating procedure (SOPs)
- Review the validation protocol
- Collect appropriate specimens for the studies
- Perform testing
- Analyze data

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Specimens, Instruments, and Reagents

Specimens

EDTA, heparin, or CPD anti-coagulated peripheral blood

All testing done by BD Biosciences was performed using EDTA, heparin, and CPD anticoagulated samples. Any claims for stability are based on the use of EDTA, heparin, and CPD as an anticoagulant.

Testing has demonstrated that HLA-B27 expression is decreased over time in acid citrate dextrose solution B (ACD-B) blood collection tubes. This decrease can lead to incorrect results, and therefore ACD-B tubes are not recommended for sample collection.

BD instruments and reagents

BD FACSCanto™ II cytometer

BD FACS™ 7-color setup beads (Catalog No. 335775)

BD™ HLA-B27 Kit (Catalog No. 340183)

Falcon® test tubes (from Corning) or equivalent

BD FACS™ Shutdown solution (Catalog No. 334224)

BD FACS™ Clean solution (Catalog No. 340345)

BD FACSFlow™ sheath fluid (Catalog No. 342003)

BD CellWASH™ (Catalog 349524) or a wash buffer of phosphate buffered saline (PBS) with 0.1% sodium azide

Other reagents required

Deionized or distilled water, reagent grade
1% paraformaldehyde solution in PBS with 0.1% sodium azide

Statistical software

Data Innovations EP Evaluator® software or equivalent

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Agreement Protocol

Reference: CLSI document EP-12

The HLA-B27 assay is qualitative—either positive or negative. Agreement between the reference and BD assay needs to be maintained.

Specimens

- Include a minimum of 20 fresh samples in any of the anticoagulants to be tested. Samples need to include known HLA-B27 positive, HLA-B7 positive (HLA-B27 equivocal), and HLA-B27 negative patients. Known HLA-B7 samples are determined by PCR or another mono-specific antibody test.
- Make sure that each sample is less than 24 hours old (since draw).
- Set up and run each sample on both the reference method and the BD method within sample stability as listed in the Technical Data Sheet (TDS).

Procedure

1. Set up the flow cytometer according to *HLA-B27 SOP 1: BD FACSCanto™ II Cytometer Startup Procedure*.
2. Prepare samples according to the TDS. Include controls in every run.
3. Acquire the samples on the cytometer.
4. Review all results and adjust the lymphocyte gate as needed.
5. Enter the results in EP Evaluator or equivalent software for the BD platform and the reference method.

Analysis of results

Agreement is calculated as follows:

$$100\% \times \frac{(positive\ on\ both\ platforms\ + negative\ on\ both\ platforms)}{(total\ number\ of\ samples)}$$

Print the data chart and agreement chart, and put the materials in the validation notebook.

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Performance specifications

Agreement between the BD FACSCanto II and the BD FACSCanto™ system, BD HLA-B27 kit, TDS 23-2563-13

Agreement study[a,b]

Test method (BD HLA-B27 system on BD FACSCanto II system)	**Comparative method (BD HLA-B27 system on BD FACSCanto system)**		
	Positive	Negative	Total
Positive	54.5	0	54.5
Negative	0	70.5	70.5
Total	54.5	70.5	125

a. Overall agreement is 100%.

b. Both replicates for one sample agreed between the test and comparative methods but disagreed within the method itself (one replicate positive and one replicate negative on each system within two channels).

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Intra-assay (Within-run) Precision Verification Protocol

Reference: CLSI document EP-15

Precision is defined as the dispersion of replicate measurements. Precision is expressed quantitatively using the standard deviation (SD) of the LMF (Log Median Fluorescence).

In general, precision assessment will require a minimum of five to ten samples, five positive and five negative for the HLA-B27 antigen. Samples were run in duplicate for two days, two runs each day, using multiple instruments and operators, if available. Intra-assay precision is defined as replicate measurements that are tested in one run. The SD of the HLA-B27 FITC LMF was calculated.

Specimens

- Include a minimum of ten samples, five HLA-B27 positive and five HLA-B27 negative.
- Each peripheral blood sample should be fresh, less than two hours old (since draw).

Procedure

1. Set up the flow cytometer according to *HLA-B27 SOP 1: BD FACSCanto™ II Cytometer Startup Procedure*.
2. Prepare samples according to the Technical Data Sheet (TDS) or *BD HLA-B27 Application Guide for BD FACSCanto Flow Cytometers* or *HLA-B27 SOP 3: Immunofluorescent Labeling of Whole Blood with BD™ HLA-B27 Reagents, BD FACS™ SPA III Preparation*. Include controls in every run.
3. Acquire the samples on the cytometer.
4. Review all results and adjust the lymphocyte gate as needed.
5. Enter the results in EP Evaluator or equivalent software for the BD platform and the reference method.

Analysis of results

When all the results have been entered into EP Evaluator or the equivalent software, calculate the HLA-B27 FITC LMF SD.

Check that the SD values agree with the performance specifications in the following charts and in the TDS. If there are any highly discrepant values, check that there are no problems with specimen identification, data entry errors, or any method errors in the preparation or running of the samples.

Print the data chart and associated graphs and put the materials in the validation notebook.

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Performance specifications for within-run precision

Within-run precision on the BD FACSCanto II system, BD HLA-B27 kit, TDS 23-2563-13

Precision study

Precision	SD of HLA-B27 FITC LMF
Within run	0.62

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Inter-Assay (Between-run) Precision Protocol

Reference: CLSI document EP-12

Inter-assay precision is defined as replicate measurements that are tested on multiple runs. At least five HLA-B27 positive and five HLA-B27 negative samples should be set up in duplicate and acquired in multiple runs, preferably by different technologists.

Specimens

- Known HLA-B27 positive samples
- Known HLA-B27 negative samples

Procedure

1. Set up the flow cytometer according to *HLA-B27 SOP 1: BD FACSCanto™ II Cytometer Startup Procedure*.
2. Prepare samples according to the Technical Data Sheet (TDS) or *BD HLA-B27 Application Guide for BD FACSCanto Flow Cytometers* or *HLA-B27 SOP 3: Immunofluorescent Labeling of Whole Blood with BD™ HLA-B27 Reagents, BD FACS™ SPA III Preparation*. Include controls in every run.
3. Acquire the samples on the cytometer.
4. Review all results and adjust the lymphocyte gate as needed.
5. Enter the results in EP Evaluator or equivalent software for the BD platform and the reference method.

Analysis of results

When all the results for each analyte have been entered into EP Evaluator or equivalent software, calculate the SD of the HLA-B27 FITC LMF.

Check that the SD value agrees with the performance specifications in the following charts and the TDS. If there are any highly discrepant values, check that there are no problems with specimen identification, data entry errors, or any method errors in the preparation or running of the samples.

Print the data chart and associated graphs and put the materials in the validation notebook.

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Performance specifications

Overall precision on the BD FACSCanto II system, BD HLA-B27 kit, TDS 23-2563-13

Precision Study—overall

Precision	SD of HLA-B27 FITC LMF
Between runs	0.99
Between days	0.54
System total	1.18

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Interference Protocol

Reference: CLSI document EP-17

Procedure

1. Check the Technical Data Sheet (TDS) for a list of interfering substances.
2. List the interfering substances in the SOP with appropriate plans of action when present.
    - Do not use previously fixed and stored specimens.
    - Any samples which are clotted or hemolyzed should be rejected for testing.
    - Whole blood samples refrigerated before staining can give aberrant results.
    - Reject samples with less than 1 mL of whole blood in the collection tube.

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Carryover Protocol

Reference: CLSI document EP-17

Specimens

Known HLA-B27 positive and HLA-B27 negative samples

Procedure

1. Set up the flow cytometer according to *HLA-B27 SOP 1: BD FACSCanto™ II Cytometer Startup Procedure*.
2. Prepare at least five replicates of a known HLA-B27 positive sample according to the Technical Data Sheet (TDS) or *BD HLA-B27 Application Guide for BD FACSCanto Flow Cytometers* or *HLA-B27 SOP 3: Immunofluorescent Labeling of Whole Blood with BD™ HLA-B27 Reagents, BD FACS™ SPA III Preparation*.
3. Acquire a known HLA-B27 positive sample followed by a known HLA-B27 negative sample, alternating five times on the cytometer.
4. Review all results and adjust the lymphocyte gate as needed.
5. Enter the HLA-B27 FITC LMF results in EP Evaluator or equivalent software for the HLA-B27 negative samples. This value should compare to an HLA-B27 negative sample acquired ten times.

Analysis of results

When all the HLA-B27 LMF results have been entered into EP Evaluator or equivalent software, compare the average results with those obtained by acquiring a known HLA-B27 sample ten times. The results for the carryover tubes should be within the SD for the HLA-B27 FITC LMF as previously determined. Verify that there are no entry errors or mistakes in the analysis to account for the differences shown.

Print the data chart and comparison of the HLA-B27 LMF as determined by carryover and precision testing and put the materials in the validation notebook.

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Linearity Verification Protocol

Reference: CLSI document EP-6

The purpose of this protocol is to verify that the assay will produce an accurate result when the WBC count is between 3.5 and 9.4 x 10^3 WBC/µL. WBC values greater than 9.4 x 10^3 may produce results which do not meet the SD limits as established by the Technical Data Sheet.

The software requires that at least 2% of the events collected be T lymphocytes, CD3 positive, and have adequate separation between the CD3-positive and CD3-negative populations.

Specimens

A sample containing a WBC count within the limit specified should be stained. Two samples with a WBC count greater than the stated upper limit should be stained, the first as undiluted and then the second as a 1:2 dilution with PBS with 0.1% sodium azide to bring it within the stated range.

Perform the dilution of the higher than stated upper limit WBC samples in the following manner:

1. Make two-fold serial dilutions with PBS with 0.1% sodium azide such that the WBC count is diluted to approximately the upper limit of the assay as stated in the TDS.

Tube	Dilution factor	Expected CD45 counts	Volume of PBS	Volume of previous dilution
1	neat or undiluted	Greater than 9.4 x 10^3 WBC	0 µL	800 µL of sample
2	1:2	Less than 9.4 x 10^3 WBC	800 µL	800 µL of neat or undiluted

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Procedure

1. Set up the flow cytometer according to *HLA-B27 SOP 1: BD FACSCanto™ II Cytometer Startup Procedure*.
2. Create serial dilutions of the samples to be tested.
3. Prepare samples according to the Technical Data Sheet (TDS) or *BD HLA-B27 Application Guide for BD FACSCanto Flow Cytometers* or *HLA-B27 SOP 3: Immunofluorescent Labeling of Whole Blood with BD™ HLA-B27 Reagents, BD FACS™ SPA III Preparation*.
4. Acquire samples on the cytometer.
5. Review all results and adjust the lymphocyte gate as needed.
6. Enter the results in EP Evaluator or equivalent software for the actual value of each dilution and the predicted value of each dilution. If there are any highly discrepant values, check that there are no problems with specimen identification or any other method errors.

Analysis of results

Check that the regression values (r^2) of at least 95% for the HLA B27 LMF meet the statistical significance requirements of your laboratory.

Print the data chart and put the materials in the validation notebook.

Performance specifications

Linearity for BD HLA-B27 kit, TDS 23-2563-13:

Linearity for the BD HLA-B27 kit was assessed for the BD FACSCanto II system within a WBC concentration from 3.5 to 9.4 x 10^4 WBC/µL.

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Stability Study Information

All stability testing done by BD Biosciences and clinical trial sites, and sample stability was determined to be up to 48 hours when held at room temperature (20℃ to 25℃). Once stained, the samples are stable up to 24 hours at 2℃ to 8℃.

In some cases samples may be received beyond the manufacturer's stated stability testing specifications. Extended stability can be tested and samples used beyond the manufacturer's timing with documentation. If the SD of the HLA-B27 FITC LMF exceeds the inter-assay precision when compared to time 0, then the stability of the sample is in question.

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