IOTest®

HLA-B27-FITC/ HLA-B7-PE

REF A07739
50 tests ; 1 mL
20 µL / test

BECKMAN COULTER

IOTest
Conjugated Antibodies

IVD CE

ENGLISH

	Specification of constituent 1	Specification of constituent 2
Specificity	HLA-B27	HLA-B7
Clone	HLA-ABC-m3	BB7.1
Hybridoma	NS1 x Balb/c	NS1 x Balb/c
Immunogen	HLA-B27 antigen partially purified, extracted from the Bordin line (HLA-B27$^+$)	HLA-A2, B7 antigen partially purified, obtained by papain cleavage
Immunoglobulin	IgG2a	IgG1
Species	Mouse	Mouse
Source	Ascites	Ascites
Purification	Chromatography	Chromatography
Fluorochrome	Fluorescein isothiocyanate (FITC)	R Phycoerythrin (PE)
λ excitation	488 nm	488 nm
Emission peak	525 nm	575 nm
Buffer	PBS pH 7.2 plus 2 mg / mL BSA and 0.1% NaN_3	

USE

This fluorochrome-conjugated antibody mixture permits the identification and numeration of cell populations expressing the HLA-B27 antigen present in human biological samples using flow cytometry.
The role of the HLA-B7 specificity monoclonal antibody is to demonstrate the presence of this antigen, the main cause of the cross-reaction of the clone recognizing the HLA-B27 antigen (1, 2).
Not for use in the determination of HLA-B tissue group.

PRINCIPLE

This test is based on the ability of specific monoclonal antibodies to bind to the antigenic determinants expressed by leucocytes on the surface.
Specific staining of the leucocytes is performed by incubating the sample with the IOTest reagent. The red cells are then removed by lysis and the leucocytes, which are unaffected by this process, are analyzed by flow cytometry.
The flow cytometer measures light diffusion and the fluorescence of cells. It makes possible the delimitation of the population of interest within the electronic window defined on a histogram, which correlates the orthogonal diffusion of light (Side Scatter or SS) and the diffusion of narrow-angle light (Forward Scatter or FS). Other histograms combining two of the different parameters available on the cytometer can be used as supports in the gating stage depending on the application chosen by the user.
The fluorescence of the delimited cells is analyzed in order to distinguish the positively-stained events from the unstained ones. In this test, in contrast to the great majority of other cytometry tests, HLA-B27 phenotyping is confirmed by comparing the intensities of fluorescence of the test sample in relation to the fluorescence intensities of the certified samples HLA-B27$^+$ and HLA-B27$^-$.

EXAMPLES OF CLINICAL APPLICATIONS

Antigens HLA-B27 and HLA-B7 belong in the cross-reaction group B7 (CREG HLA-B7). Among the other HLA-B antigens belonging to CREG HLA-B7, B42 and B22 can be cited (subdivided into B54, B55 and B56), B40 (subdivided into B60 and B61), as well as B41, B47 and B13 (3). The HLA-ABC-m3 monoclonal antibody recognizes the HLA-B27 antigen. It crosses with the HLA-B7 antigen (4) and can also become fixed, but in a less refined manner, to the other antigens of CREG HLA-B7. The BB7.1 monoclonal antibody recognizes the HLA-B7 antigen (5, 6). Though highly specific, it crosses with the HLA-B42 antigen (2). The HLA-B27 antigens, for which 15 alleles (B*2701-15) have been found (7), and HLA-B7, for which 11 alleles (B*0702-12) have been found (7), are expressed in 7% and 22% of individuals of Caucasian origin (8, 9).
This mixture permits the characterization of HLA-B27 specificity in the HLA class I allotype in patients suffering from inflammatory disorders affecting the sacroiliac and intervertebral joints. This finding aids in the diagnosis of ankylosing spondylitis 90% of sufferers of which express the HLA-B27 antigen, versus 7% in the normal population (10 – 13).

STORAGE AND STABILITY

The conjugated liquid forms must be kept at between 2 and 8°C and protected from light, before and after the vial has been opened.
Stability of closed vial: see expiry date on vial.
Stability of opened vial: the reagent is stable for 90 days.

PRECAUTIONS

1. Do not use the reagent beyond the expiry date.
2. Do not freeze.
3. Let it come to room temperature (18 – 25°C) before use.
4. Minimize exposure to light.
5. Avoid microbial contamination of the reagents, or false results may occur.
6. Antibody solutions containing sodium azide (NaN_3) should be handled with care. Do not take internally and avoid all contact with the skin, mucosa and eyes.
   Moreover, in an acid medium, sodium azide can form the potentially dangerous hydrazoic acid. If it needs to be disposed of, it is recommended that the reagent be diluted in a large volume of water before pouring it into the drainage system so as to avoid the accumulation of sodium azide in metal pipes and to prevent the risk of explosion.
7. All blood samples must be considered as potentially infectious and must be handled with care (in particular: the wearing of protective gloves, gowns and goggles).
8. Never pipette by mouth and avoid all contact of the samples with the skin, mucosa and eyes.
9. Blood tubes and disposable material used for handling should be disposed of in ad hoc containers intended for incineration.

SAMPLES

This test can just as readily be undertaken on whole blood as on cells separated on a density gradient. Venous blood or bone marrow samples must be taken using sterile tubes containing an EDTA salt as the anticoagulant. The use of other anticoagulants is not recommended.
The samples should be kept at room temperature (18 – 25°C) and not shaken. The sample should be homogenized by gentle agitation prior to taking the test sample.
The samples must be analyzed within 24 hours of venipuncture.

METHODOLOGY

NECESSARY MATERIAL NOT SUPPLIED

- Sampling tubes and material necessary for sampling.
- Automatic pipettes with disposable tips for 20, 100 and 500 µL.
- Plastic haemolysis tubes.
- Calibration beads: Flow-Set™ Fluorospheres (Ref. 6607007).
- Red cell lysis reagent with washing stage after lysis. For example:
  IOTest 3 Lysis Solution (Ref. A07799).
- Fixation reagent: IOTest 3 Fixative Solution (Ref. A07800).
- Isotypic control: A mixture of IgG2a-FITC and IgG1-PE, both from mouse.
- Compensation adjustment reagent: A mixture of CD8-FITC and CD4-PE monoclonal antibodies.
- Control blood: IMMUNO-TROL™ Control Cells (Ref. 6607077).
- Buffer (PBS: 0.01 M sodium phosphate; 0.145 M sodium chloride; pH 7.2).
- Centrifuge.
- Automatic agitator (Vortex type).
- Flow cytometer.

PROCEDURE

The clinical responses given by this test depend upon the intensity of fluorescence; these being markedly dependent upon compensation adjustments, it is essential to allow with each analysis series for a tube for the verification of these adjustments. For this verification, refer to the section: **CYTOMETRIC ADJUSTMENTS.**
For each sample analyzed, in addition to the test tube, one control tube is required in which the cells are mixed in the presence of an isotypic control.

1. Add 20 µL of specific IOTest conjugated antibodies to each test tube, and to each control tube, the necessary amount of the isotypic control.
2. Add 100 µL of the test sample to the 2 tubes. Vortex the tubes gently.
3. Incubate for 15 to 20 minutes at room temperature (18 – 25°C), protected from light.
4. Then perform lysis of the red cells, if necessary, by following the recommendations of the lysis reagent used. By way of example, if one wishes to use the IOTest 3 Lysis Solution (Ref. A07799), add 2 mL of this reagent at its working concentration (1X). Vortex

immediately and incubate for 10 minutes at room temperature, protected from light. If the sample does not contain red cells, replace the lysis with 2 mL of PBS.

5. Centrifuge for 5 minutes at 300 x g at room temperature.
6. Remove the supernatant by aspiration.
7. Resuspend the cell pellet using 3 mL of PBS.
8. Repeat step 5.
9. Remove the supernatant by aspiration and resuspend the cell pellet using:

– 0.5 mL or 1 mL of IOTest 3 Fixative Solution (Ref. A07800) at its working concentration (1X), if the preparations are to be kept for more than 2 hours and for less than 24 hours,
– 0.5 mL or 1 mL of PBS without formaldehyde, if the preparations are to be analyzed within 2 hours.

NOTE: In all cases, keep the preparations between 2 and 8°C and protected from light.

CYTOMETRIC ADJUSTMENTS

This cytometric adjustment procedure should be fully carried out only at the time of the first use of the reagent. On the other hand, the compensation adjustments, which are described here, need to be repeated prior to each series of analyses.

NECESSARY MATERIAL NOT SUPPLIED

For the first use of this reagent, adjustment of the cytometer can only be carried out on blood for which the HLA phenotype is fully documented. Obtain samples of the following blood:

- Whole blood with an HLA-B27$^+$ B7$^-$ phenotype.
- Whole blood with an HLA-B27$^+$ B7$^+$ phenotype.
- Whole blood with an HLA-B27$^-$ B7$^+$ phenotype.
- Whole blood with an HLA-B27$^-$ B7$^-$ phenotype.

PREPARATION OF SAMPLES REQUIRED FOR ADJUSTMENTS

Tubes for blood with a known HLA typing

For each of these 4 samples prepare a test tube and a control tube by following the staining procedures in section: METHODOLOGY/ PROCEDURE.

Tube for compensation adjustments

Prepare a tube for compensation adjustment by staining the blood of a healthy donor or the Immuno-Trol (Ref. 6607077) control blood with the CD8-FITC/CD4-PE mixture by following the procedure given in the section: METHODOLOGY / PROCEDURE, in which, firstly the tube for the isotypic control is omitted and secondly, the volume of antibody used is that recommended by the manufacturer of the mixture for 100 µL of blood.

PHOTOMULTIPLICATOR ADJUSTMENT

Analyze the blood control tube HLA-B27$^-$ B7$^-$ and adjust the FITC and PE signals (logarithmically amplified) so that the intensity of fluorescence in a biparametric FITC histogram *versus* PE, undertaken on lymphocytes, is in the first decade counter as shown in the histogram in figure 1.

Analyze the HLA-B27$^-$ B7$^-$ blood test tube and check that the histogram obtained is comparable to that of figure 2. Adjust the quadrants so that 98% of the lymphocytic population is in quadrant 3.

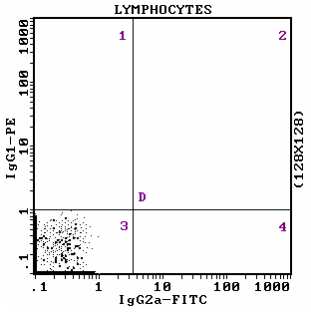

Figure 1: Control tube of HLA-B27$^-$ B7$^-$ blood

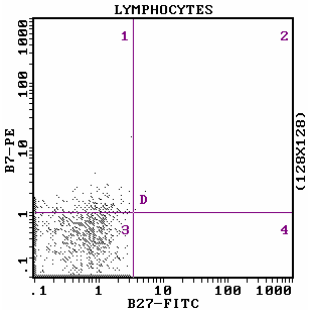

Figure 2: Test tube of HLA-B27$^-$ B7$^-$ blood

COMPENSATION ADJUSTMENTS

Analyze the tube stained with the CD8-FITC/CD4-PE mixture and adjust the fluorescence compensations such that, in the FITC *versus* PE histogram, the means of the fluorescence intensities are identical along the Y axis in quadrants 3 and 4 and identical along the X axis in quadrants 1 and 3.

CONTROL OF FLUORESCENCE THRESHOLDS

This control is carried out with blood of known HLA typing.

Sample HLA-B27$^+$ B7$^-$

Analyze the HLA-B27$^+$ B7$^-$ blood control tube. The histogram obtained must be comparable to that of Figure 1.

Analyze the HLA-B27$^+$ B7$^-$ blood test tube. The histogram obtained must be comparable to that of Figure 3 where all the lymphocytes are found in quadrant 4.

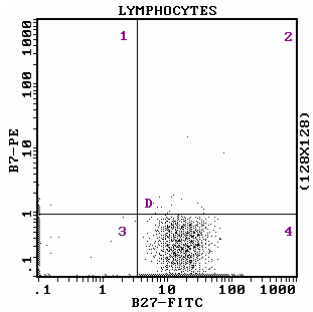

Figure 3: Test tube of HLA-B27$^+$ B7$^-$ blood

Sample HLA-B27$^-$B7$^+$

Analyze the HLA-B27$^-$ B7$^+$ blood control tube. The histogram obtained must be comparable to that of figure 1.

Analyze the HLA-B27$^-$ B7$^+$ blood test tube. The histogram obtained should resemble that of Figure 4.

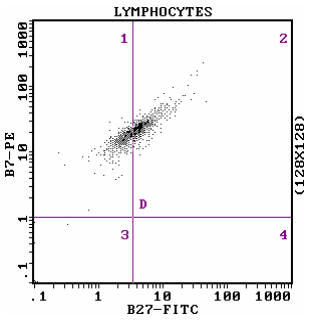

Figure 4: Test tube of HLA-B27$^-$ B7$^+$ blood

The lymphocytic population is straddled between quadrants 1 and 2. This situation is entirely normal and no attempt should be made to try and compensate for it. It is due to the fact that the HLA-ABC-m3 monoclonal antibody crosses with the HLA-B7 phenotype. A fraction of the HLA-B7 epitopes is occupied by the HLA-ABC-m3 antibody. A lymphocytic population completely contained within quadrant 1 is also possible. It corresponds to the case in which the HLA-ABC-m3 monoclonal antibodies do not cross with the HLA-B7 epitopes.

Sample HLA-B27$^+$B7$^+$

Analyze the HLA-B27$^+$ B7$^+$ blood control tube. The histogram obtained should be comparable with that of Figure 1.

Analyze the HLA-B27$^+$ B7$^+$ blood test tube. The histogram obtained should be comparable to that of Figure 5.

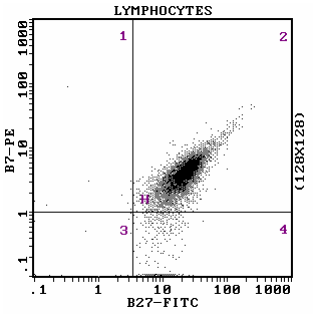

Figure 5: Test tube of HLA-B27$^+$ B7$^+$ blood

Sample HLA-B27$^-$ B7$^-$

Analyze the control tube of HLA-B27$^-$ B7$^-$ blood. The histogram obtained should be comparable to that of Figure 1.

Analyze the test tube of HLA-B27$^-$ B7$^-$ blood. The histogram obtained should be comparable to that of figure 2.

INTERPRETATION OF THE RESULTS

All lymphocytic populations completely contained within quadrant 3 can be considered as being HLA-B27$^-$ (and HLA-B7$^-$).

All lymphocytic populations completely contained within quadrant 1 can be considered as being HLA-B27$^-$ (and HLA-B7$^+$).

All lymphocytic populations completely contained within quadrant 4 can be considered to be HLA-B27$^+$– providing the quadrants have been positioned, not on an isotypic control, but over an HLA-B27$^-$ B7$^-$ sample.

All lymphocytic populations straddling quadrants 3 and 4 have a high probability of being HLA-B27$^+$ (and HLA-B7$^-$).

However, a confirmatory microlympho-cytotoxicity test or using PCR is essential.

All lymphocytic populations straddling quadrants 1 and 2 have a high probability of being HLA-B27$^-$ (and HLA-B7$^+$).

However, a confirmatory microlympho-cytotoxicity test or using PCR is essential.

All lymphocytic populations contained within quadrant 2 have a high probability of being HLA-B27$^+$ (and HLA-B7$^+$).

However, a confirmatory microlympho-cytotoxicity test or using PCR is essential.

PERFORMANCE

SPECIFICITY

The specificity of the HLA-ABC-m3 monoclonal antibody vis-à-vis the HLA-B27 antigen has been studied by Trapani, J.A. *et al*. (4) and that of the BB7.1 monoclonal antibody by Brodsky, F.M. *et al*. (5).

LINEARITY

A linearity test of staining has no biological significance as in a given individual either 100% of cells are HLA-B27$^+$ or 100% are HLA-B27$^-$. On the other hand, such a reagent must show in HLA-B27$^-$B7$^-$ donors a mean fluorescence intensity (MFI) for lymphocytes, both with regard to HLA-B27 specificity as well as to HLA-B7 specificity, which is much lower to that obtained in HLA-B27$^+$ B7$^+$ donors. This is reflected by the MFI$^+$/ MFI$^-$ ratios calculated from the mean of 12 determinations of MFI from an HLA-B27$^+$ B7$^+$ (MFI$^+$) donor and from an HLA-B27$^-$ B7$^-$ (MFI$^-$) donor shown in the following table.

Lymphocytes	Number	Mean (MFI)	CV (%)	MFI$^+$/ MFI$^-$ ratio
HLA-B27$^+$	12	29.6	2.2	114
HLA-B27$^-$	12	0.26	2.4	
HLA-B7$^+$	12	33.9	1.7	168
HLA-B7$^-$	12	0.20	1.7	

EXPECTED VALUES

Non applicable

INTRA-LABORATORY REPRODUCIBILITY

As the allocation of a subject to the HLA B27 specificity group, being based upon the measurement of the intensity of fluorescence of each lymphocyte analyzed and not on the percentage of stained lymphocytes, the study of the intra-laboratory reproducibility is based on the MFI of this population.

On the same day and using the same cytometer, 12 measurements of the MFI of lymphocytes from an HLA-B27$^+$ B7$^+$ donor were undertaken. The results obtained are summarized in the following table:

Lymphocytes	Number	Mean (MFI)	SD	CV (%)
HLA-B27	12	29.6	0.65	2.2
HLA-B7	12	33.9	0.56	1.7

INTER-LABORATORY REPRODUCIBILITY

On the same day and for the same positive target (HLA-B27$^+$B7$^+$ lymphocytes), 12 measurements of the MFI of the lymphocytes were carried out by two technicians and the preparations analyzed using two different cytometers. The results obtained are summarized in the following tables:

Cytometer n° 1:

Lymphocytes	Number	Mean (MFI)	SD	CV (%)
HLA-B27	12	29.6	0.65	2.2
HLA-B7	12	33.9	0.56	1.7

Cytometer n° 2:

Lymphocytes	Number	Mean (MFI)	SD	CV (%)
HLA-B27	12	23.7	0.58	2.5
HLA-B7	12	15.2	0.40	2.6

LIMITATIONS OF THE TECHNIQUE

1. Flow cytometry may produce false results if the cytometer has not been aligned perfectly, if fluorescence leaks have not been correctly compensated for and if the regions have not been carefully positioned.
2. This reagent must not be diluted, aliquoted or frozen. Do not use beyond the expiry date shown on the flask.
3. Phycoerythrin (PE) is sensitive to light and all incubations must take place away from light.
4. It is preferable to use a RBC lysis technique with a washing step as this reagent has not been optimized for "no wash" lysis techniques.
5. Accurate and reproducible results will be obtained as long as the procedures used are in accordance with the technical insert leaflet and compatible with good laboratory practices.
6. The conjugated antibodies of this reagent are calibrated so as to offer the best specific signal/non-specific signal ratio. Therefore, it is important to adhere to the reagent volume/sample volume ratio in every test.
7. In the case of a hyperleucocytosis, dilute the blood in PBS so as to obtain a value of approximately 5 x 10^9 leucocytes/L.
8. In certain disease states, such as severe renal failure or haemoglobinopathies, lysis of red cells may be slow, incomplete or even impossible. In this case, it is recommended to isolate mononucleated cells using a density gradient (Ficoll, for example) prior to staining.
9. The demonstration of an HLA-B27 phenotype in a patient does not signify that the latter is suffering from an ankylosing spondylitis. Conversely, only 90% of affected patients are HLA-B27$^+$.

POSSIBLE INTERFERENCES

(CROSS REACTIONS)

1. The HLA-ABC-m3 monoclonal antibody, which recognizes the HLA-B27 antigen, crosses with the HLA-B7 antigen (4) and, to a lesser extent, with CREG HLA-B7 antigens.
2. On the other hand, the BB7.1 monoclonal antibody, which recognizes the HLA-B7 antigen, crosses with the HLA-B42 antigen HLA-B42 (2, 5).
3. The demonstration by the present reagent of a double HLA-B27$^+$ and HLA-B7$^+$ related positivity may be the result of different expressions:

- the heterozygotic expression of the HLA-B27 / HLA-B7 genotype,
- the heterozygotic expression of the HLA-B7 / HLA-BY genotype or,
- the homozygotic expression of the HLA-B7 / HLA-B7 genotype.

A confirmatory test using microlymphocytotoxicity or PCR must therefore be undertaken.

MISCELLANEOUS

See the Appendix for references.

TRADEMARKS

MANUFACTURED BY:

IMMUNOTECH SAS
a Beckman Coulter Company
130 avenue de Lattre de Tassigny
B.P. 177 – 13276 Marseille Cedex 9
France
Customer Services: (33) 4 91 17 27 27

www.beckmancoulter.com

APPENDIX TO REF A07739

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