IOTest®3
CD41-FITC / Glycophorin A-PE / CD45-ECD

REF A07723
25 tests; 0.5 mL
20 µL / test

BECKMAN COULTER™

IOTest 3
Conjugated Antibodies

IVD CE

ENGLISH	Specifications of constituent 1	Specifications of constituent 2	Specifications of constituent 3
Specificity	CD41	Glycophorin A	CD45
Clone	P2	11E4B-7-6 (KC16)	J33
Hybridome	SP2/0-Ag14 x Balb/c	NS/1-Ag4 x Balb/c	NS1 x Balb/c
Immunogen	Human platelets	Human blood cells	LAL Laz 221 cell line
Immunoglobulin	IgG1	IgG1	IgG1
Species	Mouse	Mouse	Mouse
Source	Ascites	Ascites	Ascites
Purification	Chromatography on Protein A	Chromatography on Protein A	Chromatography on Protein A
Fluorochrome	Fluorescein isothiocyanate (FITC)	R Phycoerythrin (PE)	R Phycoerythrin-Texas Red®-X (ECD™)
λ excitation	488 nm	488 nm	488 nm
Emission peak	525 nm	575 nm	613 nm
Buffer	Buffer (PBS pH 7.2) plus 2 mg / mL BSA and 0.1% NaN_3		

USE

This fluorochrome-conjugated antibody mixture is suitable for multiparametric analysis using flow cytometry. It permits the detection of the expression of CD41, Glycophorin A and CD45 antigens in cells of hematopoietic origin.

PRINCIPLE

This test is based on the ability of specific monoclonal antibodies to bind to the antigenic determinants expressed by certain cells of hematopoietic origin.
Specific staining is performed by incubating the sample with the IOTest 3 reagent. The mature red cells are then removed by lysis and the leucocytes and hematopoietic progenitors, which are unaffected by this process, are analyzed by flow cytometry.
The flow cytometer analyzes light diffusion and the fluorescence of cells. It makes possible the localization of cells within the electronic window defined on a histogram, which correlates the orthogonal diffusion of light ("Side Scatter" or SS) with ECD fluorescence, corresponding to CD45 staining. Other histograms combining two of the different parameters available on the cytometer, are also used the electronic gating stage. The cell population thus gated is subdivided into sub-populations, using the two other fluorescences.
In this way, the positively-stained cells are distinguished from the unstained cells. The results are expressed as a percentage of fluorescent cells in relation to all the events acquired by the electronic gating.

EXAMPLES OF CLINICAL APPLICATIONS

Analysis of the expression of the CD41 antigens and Glycophorin A (Glyco A) on the surface of blast cells permits the differentiation of acute myeloid leukaemias of megacaryocyte origin (AML) from AMLs of erythrocyte origin (1).
Blast cells are targeted using a Side Scatter histogram versus CD45 ECD (2-4). A slight to average expression by the CD45 antigen is characteristic of blast cells of myeloid origin (in AML), whereas no to weak expression is observed on the surface of blast cells of lymphoid origin (in acute lymphoblastic leukaemias, ALL) (2).
Type M7 AMLs of megacaryocyte origin are $CD41^+$, Glyco A^+ and $CD45^{weak}$, whereas erythroleukaemias (type M6 AMLs) are $CD41^-$, Glyco A^+ and $CD45^{weak}$ (1, 5-7).

STORAGE AND STABILITY

The conjugated liquid forms must be kept at between 2 and 8°C and away 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 remains stable for 90 days.

PRECAUTIONS

1. Do not use the reagent beyond the expiry date.
2. Do not freeze.
3. Let it warm up 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.

SPECIMENS

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 samples should be homogenised by means of gentle agitation prior to taking the test sample.
The samples should be analyzed within 24 hours of taking them.

METHODOLOGY

NECESSARY MATERIAL NOT SUPPLIED

- Sampling tubes and material necessary for sampling.
- Automatic pipettes with disposable tips to take 20, 100 and 500 µL.
- Plastic haemolysis tubes.
- Calibration beads: Flow-Set™ Fluorospheres (Ref. 6607007).
- To obtain optimal results, the following reagents are recommended:
  - Lysing reagent: IOTest 3 Lysis Solution (Ref. A07799).
  - Fixation reagent: IOTest 3 Fixative Solution (Ref. A07800).
  - One of the following IOTest 3 negative controls:
    Neg.Ctrl.-FITC /Neg.Ctrl.-PE/CD45-ECD (Ref. A07729) or
    Neg.Ctrl.-FITC /Neg.Ctrl.-PE/Neg.Ctrl.-ECD (Ref. A07732).
- Buffer (PBS: 0.01 M sodium phosphate; 0.145 M sodium chloride; pH 7.2).
- Centrifuge.
- Automatic agitator (Vortex type).
- Flow cytometer.

PROCEDURE

For each sample analyzed, in addition to the test tube, one control tube is required in which the cells are mixed with the selected IOTest 3 negative control (Ref. A07729 or A07732).

1. Add 20 µL of specific IOTest 3 conjugated antibodies to each test tube, and 20 µL of the appropriate negative control to each control tube.
2. Add 100 µL of the test sample to the 2 tubes. Vortex the tubes slowly.
3. Incubate for 15 to 20 minutes at room temperature (18-25°C), protected from light.
4. Then perform, if necessary, lysis of the red cells by adding 2 mL of IOTest 3 Lysis Solution (Ref. A07799) 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, add 2 mL of PBS.
5. Centrifuge at 300 x g for 5 minutes at room temperature.
6. Remove the supernatant by aspiration.
7. Resuspend the cell pellet using 3 mL of PBS.
8. Repeat stage 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.

PERFORMANCE

SPECIFICITY

The CD41 molecule is expressed by platelets, megacaryocytes and by a sub-population of CD34+ cells (8).
The P2 monoclonal antibody (mAb) was assigned to CD41 during the 5th HLDA Workshop on Human Leucocyte Differentiation Antigens, Boston, USA, in 1993 (WS Code: P086 and BP335, Section M and BP respectively) (9).
Glycophorin A is the main protein expressed by red blood cells and red blood cell precursors, including the pro-erythroblasts and reticulocytes (10-12).

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The mAb 11E4B-7-6 (KC16) reacts with the 27-39 terminal N amino acid sequence of Glycophorin A and does not recognize Glycophorin B (11).
The CD45 molecule is expressed on the surface of all human leucocytes, but is absent from mature red blood cells (13). The density of expression on lymphocytes is greater than that observed for monocytes and neutrophils (14).
The mAb J33 was assigned to CD45 during the 3rd HLDA Workshop in Oxford, England, in 1986 (WS Code: 818, Section NL) (15).

LINEARITY

To test the linearity of staining for the specificities of this reagent, a positive cell line and a negative cell line were mixed in different proportions with a constant final cell quantity, so that the positive /negative line ratio of the mixture ranged from 0 to 100%.
Aliquots were stained using the procedure described above and linear regression between the expected values and the observed values was calculated. The parameters of the equation of the linear regression may be used to determine the coefficient of linearity as well as the range of measurement for each specificity.

Specificity	Linear regression	Linearity (R^2)	Range (%)
CD41	Y=0.97 X + 1.36	0.998	2 - 98
Glyco A	Y=0.96 X + 1.12	0.998	2 - 97
CD45	Y=1.00 X – 0.91	0.998	2 - 99

EXPECTED VALUES

Each laboratory must compile a list of reference values based upon a group of healthy donors belonging to a local population. This must be done by taking age, sex and ethnic group into account, as well as any other potential regional differences.
In our laboratories, the whole blood of 10 healthy adults was treated using the reagent described above. The results obtained in the leucocyte subpopulations of interest in these 10 donors are shown in the tables below: Since no normal leucocyte population was positive for CD41 and Glycophorin A, no expected value for these has been given.

Lymphocytes	Number	Mean (%)	SD	CV (%)
$CD45^+$	10	95.5	4.0	4.2

Monocytes	Number	Mean (%)	SD	CV (%)
$CD45^+$	10	90.2	10.3	11.4

Granulocytes	Number	Mean (%)	SD	CV (%)
$CD45^+$	10	99.8	0.11	0.1

INTRA-LABORATORY REPRODUCIBILITY

On the same day and using the same cytometer, 12 measurements of the percentage of positive cells were carried out on a target population expressing these three markers (HEL cell line). The results obtained are shown in the following table:

HEL Line	Number	Mean (%)	SD	CV (%)
$CD41^+$	12	100	0.03	0.03
Glyco A^+	12	96.2	0.36	0.40
$CD45^+$	12	100	0.00	0.00

INTER-LABORATORY REPRODUCIBILITY

On the same day and for the same population (HEL line), 12 measurements of the percentage of positive cells were performed by two technicians and the preparations were analyzed on two different cytometers. The results obtained are grouped in the following tables:

Cytometer n°1:

HEL Line	Number	Mean (%)	SD	CV (%)
$CD41^+$	12	100	0.03	0.03
Glyco A^+	12	96.2	0.36	0.40
$CD45^+$	12	100	0.00	0.00

Cytometer n°2:

HEL Line	Number	Mean (%)	SD	CV (%)
$CD41^+$	12	100	0.00	0.0
Glyco A^+	12	98.7	0.15	0.2
$CD45^+$	12	100	0.06	0.1

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. It is preferable to use a lysis technique with washing as this reagent has not been optimized for "without washing" lysis techniques.
3. 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.
4. The 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/number of cells ratio in every test.
5. 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.
6. 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.
7. CD45-negative or very weakly-positive acute lymphoblastic leukaemia have been described. For these, the lymphocytic origin of the blast cells should be confirmed using other markers.

MISCELLANEOUS

See the Appendix for references.

TRADEMARKS

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APPENDIX TO REF A07723

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