IOTest®3
HLA-DR-FITC / CD34-PE / CD45-ECD

REF A07719
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	HLA-DR	CD34	CD45
Clone	Immu357	581	J33
Hybridoma	P3-X63-Ag.8.653 x Balb/c	NSO x Balb/c	NS1 x Balb/c
Immunogen	Cell line transformed by EBV	KG-1a cell line + human $CD34^+$ leukaemia	Laz 221 cell line
Immunoglobulin	IgG1	IgG1	IgG1
Species	Mouse	Mouse	Mouse
Source	Ascites	Ascites	Ascites
Purification	Protein A affinity chromatography	Protein A affinity chromatography	Protein A affinity chromatography
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	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 HLA-DR, CD34, and CD45 on leucocytes.

PRINCIPLE
This test is based on the ability of specific monoclonal antibodies to bind to the antigenic determinants expressed by leucocytes.
Specific staining of the leucocytes is performed by incubating the sample with the IOTest 3 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 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 the fluorescence of ECD, corresponding to CD45 staining. Other histograms combining two of the different parameters available on the cytometer are also used in the 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 gating.

EXAMPLES OF CLINICAL APPLICATIONS
Analysis of the expression of antigen CD45 is useful for the phenotyping of leukaemias by defining blast cells within a window on a histogram which correlates the orthogonal diffusion of light (Side Scatter) of analysed cells with the ECD (CD45) fluorescence (1 – 3).
A slight to average expression by the CD45 antigen is characteristic of blast cells of myeloblastic origin (cases of acute myeloblastic leukaemias, AML), whereas no to weak expression will more likely be seen in blast cells of lymphoid origin (cases of acute lymphoblastic leukaemias, ALL) (1). Finally, the co-expression of HLA-DR and CD34 antigens is useful for better characterizing the immaturity of blast cells (4 – 8). In order to confirm the myeloid origin of analysed blast cells, an additional study can be carried out using the following IOTest 3 reagents: CD34-FITC / CD33-PE / CD45-ECD (Ref. A07717), CD34-FITC / CD117-PE / CD45-ECD (Ref. A07718) and HLA-DR-FITC / CD13-PE / CD45-ECD (Ref. A07720).

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.

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 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).
- 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 IOTest 3 negative control (Ref. A07729 or A07732).
1. Add 20 µL of specific IOTest 3 conjugated antibodies to each 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 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 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 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 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

Immu357 monoclonal antibody (mAb) recognizes an epitope carried by a 29 – 33 kDa monomorphic protein identified as HLA-DR.
MAb 581 was assigned to CD34 during the 5th HLDA Workshop on Human Leucocyte Differentiation Antigens held in Boston, USA, in 1993 (WS Code: MA027, Section M) (9).
MAb J33 reacts with all the isoforms of CD45 (180 to 220 kDa): it is therefore referenced as a pan-leucocyte marker. MAb J33 assigned to CD45 during the 3rd HLDA Workshop, Oxford, England, in 1986 (WS Code: 818, Section NL) (10).

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 number of cells, so that the positive line/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.

Specificity	Linear regression	Linearity (R^2)
HLA-DR	Y = 0.97 X + 1.30	0.997
CD34	Y = 0.99 X + 0.22	0.999
CD45	Y = 0.99 X + 0.16	0.999

EXPECTED VALUES

Each laboratory must compile a list of reference values based upon a group of healthy donors from the 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 samples of 50 healthy adults were treated using the reagent described above. As no leucocyte population is positive for CD34, the expected values for this specificity are not given. The mean values of the results obtained in the leucocyte sub-populations of interest are shown in the tables below:

Lymphocytes	Number	Mean (%)	SD	CV (%)
HLA-DR+	50	17.32	9.48	55
CD45+	50	94.63	3.77	4

Monocytes	Number	Mean (%)	SD	CV (%)
HLA-DR+	50	91.87	6.29	7
CD45+	50	95.67	2.88	3

Granulocytes	Number	Mean (%)	SD	CV (%)
CD45+	50	99.86	0.17	0

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 (mixture of human cell lines DAUDI and KG-1a) expressing these three markers. The results obtained are summarized in the following table:

DAUDI & KG-1a mixture	Number	Mean (%)	SD	CV (%)
HLA-DR+	12	44.65	1.03	2.30
CD34+	12	55.09	1.06	1.92
CD45+	12	99.75	0.05	0.05

INTER-LABORATORY REPRODUCIBILITY

On the same day and for the same population (DAUDI and KG-1a lines), 12 measurements of the percentage of positive cells 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:

DAUDI & KG-1a mixture	Number	Mean (%)	SD	CV (%)
HLA-DR+	12	44.65	1.03	2.30
CD34+	12	55.09	1.06	1.92
CD45+	12	99.75	0.05	0.05

Cytometer n°2:

DAUDI & KG-1a mixture	Number	Mean (%)	SD	CV (%)
HLA-DR+	12	43.63	1.02	2.3
CD34+	12	55.58	0.95	1.7
CD45+	12	99.79	0.09	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 RBC lysis technique with a washing step as this reagent has not been optimized for "no wash" 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 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.
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 advisable 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 examples and references.

TRADEMARKS

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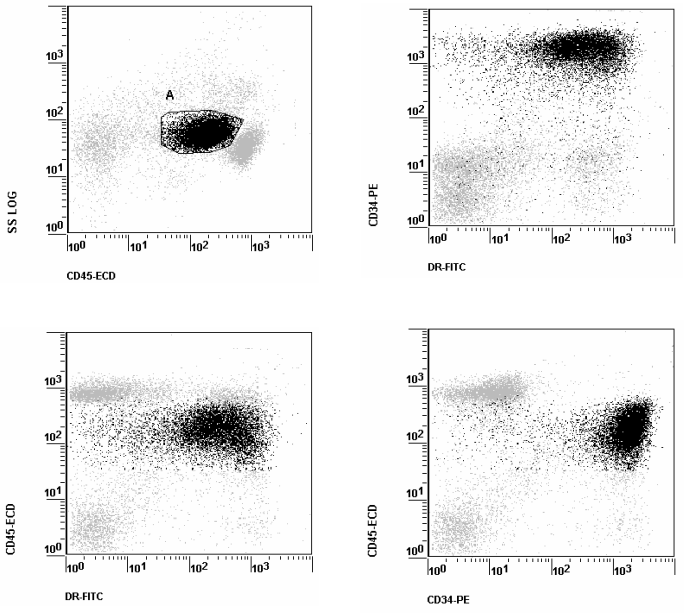
APPENDIX TO REF A07719

EXAMPLES

The 4 diagrams below are biparametric representations (Side Scatter *versus* Fluorescence Intensity or Fluorescence Intensity *versus* Fluorescence Intensity) of an Acute Myeloiblastic Leukemia specimen (AML-M0, Bone marrow aspirate). Staining is with HLA DR-FITC / CD34-PE / CD45-ECD Conjugated Antibodies (Ref. A07719). Lysis and fixation are with IOTest 3 Lysing Solution (Ref. A07799) and IOTest 3 Fixative Solution (Ref. A07800) respectively. All events acquired are shown. Region A defines the gating strategy (CD45 positive blasts cluster) used on this example. The gated events are shown in dark in all histograms.

Acquisition is with a COULTER® EPICS® XL™ flow cytometer equipped with System II™ Software. Analysis is with EXPO™ Cytometer Software (Ref. 6605434).



REFERENCES

1. Borowitz, M.J., Guenther, K.L., Shults, K.E., Stelzer, G.T., "Immunophenotyping of acute leukemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukemic blasts in three-color analysis", 1993, Am. J. Clin. Pathol., 100, 534-540.
2. Seltzer, G.T., Shults, K.E., Loken, M.R., "CD45 gating for routine flow cytometric analysis of human bone marrow specimens", 1993, Acad. Sciences, 265-280.
3. Lacombe, F., Durrieu, F., Briais, A., Dumain, P., Belloc, Bascans, E., Reiffers, J., Boisseau, M.R., Bernard, P., "Flow cytometry CD45 gating for immunophenotyping of acute myeloid leukemia", 1997, Leukemia, 11, 1878-1886.
4. Bene, M.C., Castoldi, G., Knapp, W., Ludwig, W.D., Matutes, E., Orfao, A., van't Veer, M.B., "Proposals for the immunological classification of acute leukemias", 1995, Leukemia, 9, 1783-1786.
5. Borowitz, M.J., Bray, R., Gascoyne, R., Melnick, S., Parker, J.W., Picker, L., Stetler-Stevenson, M., "U.S. Canadian consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: Data analysis and interpretation", 1997, Cytometry, 30, 236-244.
6. Stewart, C.C., Behm, F.G., Carey, J.L., Cornbleet, J., Duque, R.E., Hudnall, S.D., Hurtubise, P.E., Loken, M., Tubbs, R.R., Wormsley, S., "U.S. Canadian consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: Selection of antibody combinations", 1997, Cytometry, 30, 231-235.
7. Rothe, G., Schmitz, G., Adorf, D., Barlage, S., Gramatzki, M., Höffkes, H.G., Janossy, G., Knüchel, R., Ludwig, W.D., Nebe, T, Nerl, C., Orfao, A., Serke, S., Sonnen, R., Tichelli, A., Wörmann, B., "Consensus protocol for the flow cytometric immunophenotyping of hematopoietic malignancies", 1996, Leukemia, 10, 877-895.
8. Jenning, C.D., Foon, K.A., "Recent advances in flow cytometry: Application to the diagnosis of hematologic malignancy", 1997, Blood, 90, 2863-2892.
9. Greaves, M.F., Titley, I., Colman, S.M., Bühring, H.-J., Campos, L., Castoldi, G.L., Garrido, F., Gaudernack, G., Girard, J.-P., Inglès-Esteve, J., Invernizzi, R., Knapp, W., Lansdorp, P.M., Lanza, F., Merle-Béral, H., Parravicini, C., Razak, K., Ruiz-Cabello, F., Springer, T.A., van der Schoot, C.E., Sutherland, D.R., "CD34 cluster Workshop report", 1995, Leucocyte Typing V, White Cell Differentiation Antigens. Schlossman, S.F., et al., Eds., Oxford University Press, 840-846
10. Cobbold, S., Hale, G., Waldmann, H., "Non-lineage, LFA-1 family, and leukocyte common antigens: New and previously defined clusters", 1987, Leucocyte Typing III, White Cell Differentiation Antigens, McMichael A.J., et al., Eds., Oxford University Press, 788-803.