IOTest®3
CD34-FITC / CD33-PE / CD45-ECD

REF A07717
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	CD34	CD33	CD45
Clone	581	D3HL60.251	J33
Hybridoma	NS0 x Balb/c	NS1 x Balb/c	NS1 x Balb/c
Immunogen	KG1a cell line + human CD34+ leukaemia	HL60 cell line	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 the expression of CD34, CD33, and CD45 antigens in 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 by flow cytometry 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 analyzed cells with ECD (CD45) fluorescence (1–3). A slight to average expression by the CD45 antigen is characteristic of blast cells of myeloid origin (acute myeloblastic leukaemias, AML), whereas no to weak expression will more likely be seen in blast cells of lymphoid origin (acute lymphoblastic leukaemias, ALL) (1). Finally, the simultaneous analysis of CD34 and CD33 antigens is useful for the characterization of blast cells of myeloid origin: the degree of immaturity is defined by the expression of CD34 and the myeloid origin by the co-expression of CD33 (4 – 8).

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 the 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

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 (i.e., the equivalent of approximately 5 x 10^5 cells). 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

CD34 is a transmembrane monomeric glycoprotein with a molecular weight of approximately 110 kDa (9 – 11). It is one of the earliest markers of progenitory cells for human haematopoiesis (12, 13). This antigen is expressed by almost all haematopoietic progenitory cells as well as by pluripotent stem cells (14 – 16). The degree of CD34 antigen expression is higher in the more precocious stem cells and decreases with the advancing commitment and maturation of cells in the various haematopoietic cell lines (14, 17). In healthy subjects, the transient expression of CD34 during haemopoiesis is demonstrated by a very low proportion of positive cells in studied samples. Thus, the CD34 antigen is normally only expressed by 1 to 5 % of bone marrow or cord blood cells (14) and by 0.1 to 0.5 % of cells in the peripheral blood (18).
The expression of the molecule is also found at the vascular endothelial level (19, 20) as well as in stromal cells (and precursors) of bone marrow (21).
MAb 581 was assigned to CD34 during the 5th HLDA Workshop on Human Leucocyte Differentiation Antigens, in Boston, USA, in 1993 (WS Code: MA027, Section M) (22).

The CD33 antigen is a transmembrane monometric glycoprotein with a molecular weight of 67 kDa. This molecule is part of the sialoadhesin group: its adhesive properties depend on the presence of sialic acid (23). CD33 is expressed by haemopoietic progenitor cells of the myelo-monocytic and erythroid cell lines; it is absent from progenitor cells of the lymphoid line (23).
The CD33 antigen is expressed strongly on monocytes and weakly on circulating granulocytes.
MAb D3HL60.251 was assigned to CD33 during the 4th HLDA Workshop in Vienna, Austria, in 1989 (WS Code: 504, Section M) (23).

MAb J33 reacts with all the isoforms of CD45 (180 to 220 kDa): it is therefore referenced as a pan-leucocyte marker. J33 was assigned to the CD45 molecule during the 3rd HLDA Workshop in Oxford, England, in 1986 (WS Code: 818, Section NL) (24).

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)
CD34	Y = 0.98 X + 1.29	0.999
CD33	Y = 0.99 X + 0.58	0.999
CD45	Y = 0.99 X + 0.75	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 in these 50 donors are shown in the tables below:

Lymphocytes	Number	Mean (%)	SD	CV (%)
$CD45^+$	50	96.31	2.61	3

Monocytes	Number	Mean (%)	SD	CV (%)
$CD33^+$	50	93.29	4.63	5
$CD45^+$	50	96.23	3.02	3

Granulocytes	Number	Mean (%)	SD	CV (%)
$CD33^+$	50	99.64	0.43	0.4
$CD45^+$	50	99.87	0.18	0.2

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 (MO7E and KG1A cell lines).

MO7E + KG1A mixture	Number	Mean (%)	SD	CV (%)
$CD34^+$	12	68.39	0.44	0.6
$CD33^+$	12	27.05	0.35	1.3
$CD45^+$	12	100.00	0.00	0.0

INTER-LABORATORY REPRODUCIBILITY

On the same day and for the same population (MO7E and KG1A 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:

MO7E + KG1A mixture	Number	Mean (%)	SD	CV (%)
$CD34^+$	12	68.39	0.44	0.6
$CD33^+$	12	27.05	0.35	1.3
$CD45^+$	12	100.00	0.00	0.0

Cytometer n°2:

MO7E + KG1A mixture	Number	Mean (%)	SD	CV (%)
$CD34^+$	12	68.29	0.42	0.6
$CD33^+$	12	26.98	0.26	1.0
$CD45^+$	12	99.98	0.04	0.0

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 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 examples and references.

TRADEMARKS

The Beckman Coulter logo, COULTER, ECD, EPICS, EXPO, Flow-Set, IOTest, System II, and XL are the registered trademarks of Beckman Coulter Inc.
Texas Red is a registered trademark of Molecular Probes Inc.

MANUFACTURED BY:

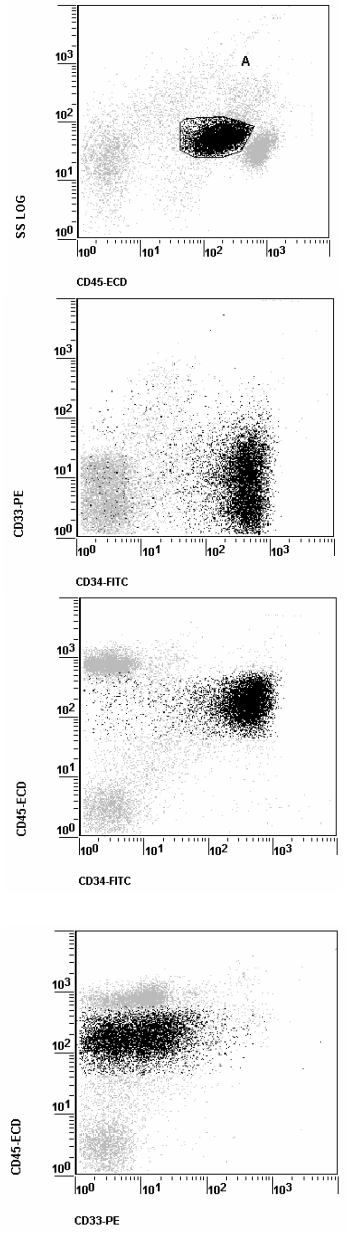
IMMUNOTECH
a Beckman Coulter Company
130 avenue de Lattre de Tassigny
B.P. 177 – 13276 Marseille Cedex 9
France
Customer Services: (33) 4 91 17 27 27
www.beckmancoulter.com

APPENDIX TO REF A07717

EXAMPLES

The 4 diagrams below are biparametric representations (Side Scatter versus Fluorescence Intensity or Fluorescence Intensity versus Fluorescence Intensity) of an Acute Myeloblastic Leukemia specimen (AML-M0, Bone marrow aspirate). Staining is with CD34-FITC / CD33-PE / CD45-ECD Conjugated Antibodies (Ref. A07717). Lysis and fixation are with IOTest 3 Lysing Solution (Ref. A07799) and IOTest 3 Fixative Solution (Ref. A07800) respectively. All acquired events 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. Sutherland, Keating, A., "The CD34 antigen: Structure, biology and potential clinical applications", 1992, J. Hematotherapy, 1, 115-129.
10. Krause, D.S., Fackler, M.J., Civin, C.I., Stratford, M., "CD34: Structure, biology and clinical utility", 1996, Blood, 87, 1-13.
11. To, L.B., Haylock, D.N., Simmons, P.J., Juttner, C.A., "The biology and clinical uses of blood stem cells", 1997, Blood, 89, 2233-2258.
12. Andrews, R.G., Singer, J.W., Bernstein, I.D., "Human hematopoietic precursors in long-term culture, Single $CD34^+$ cells that lack detectable T cell, B cells and myeloid cell antigens produce multiple colony-forming cells when cultured with marrow stromal cells", 1990, J. Exp. Med., 172, 355-358.
13. Lansdorp, P.M., Sutherland, H.J, Eaves, C.J., "Selective expression of CD45 isoforms on functional subpopulations of $CD34^+$ hemopoietic cells from human bone marrow", 1990, J. Exp. Med., 172, 363-366.
14. Civin, C.I., Strauss, L.C., Brovall, c., Jackler, M.J., Schwartz, J.F., Shaper, J.H., "Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells", 1984, J. Immunol., 133, 157-165.
15. Berenson, R.J., Bensinger, W.I., Hill, R.S., Andrews, R.G., Garcia-Lopez, J., Kalamaz, D.F., Still, B.J., Spitzer, G., Buckner, C.D., Bernstein, I.D., Thomas, E.D., "Engraftment after infusion of $CD34^+$ marrow cells in patients with breast cancer or neuroblastoma", 1991, Blood, 77, 1717-1722.
16. Terstappen, L.W.M.M., Huang, S., Safford, M., Lansdorp, P.M., Loken, M.R., "Sequential generation of hematopoietic colonies derived from single nonlineage-committed $CD34^+CD38^-$ progenitor cells", 1991, Blood, 77, 1218-1227.
17. Bender, J.G., Unverzagt, K.L., Walker, D.E., Lee, W., Van Epps, D.E., Smith, D.H., Stewart, C.C., To, L.B., "Identification and comparison of CD34 positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry", 1991, Blood, 77, 2591-2596.
18. Siena.S., Bregni, M., Brando, B., Ravignanil, F., Bonadonna, G., Gianni, A.M.,, "Circulation of $CD34^+$ hematopoietic stem cells in the peripheral blood of high dose cyclophosphamide-treated patients: Enhancement by intravenous recombinant human granulocyte macrophage colony stimulating factor", 1989, Blood, 74, 1905-1914
19. Fina, L., Molgaard, H.V., Robertson, d., Bradley, N.J., Monaghan, P., Delia, D., Sutherland, D.R., Baker, M.A., Greaves, M.F., "Expression of the CD34 gene in vascular endothelial cells", 1990, Blood, 75, 2417-2426.
20. Delia, D., Lampugnani, M.G., Resnati, M., Dejana, E., Ajello, A., Fontanella, E., Soligo, D., Pierotti, M.A., Greaves, M.F., "CD34 expression is regulated reciprocally with adhesion molecules in vascular endothelial cells in vitro", 1993, Blood, 81, 1001-1008.
21. Simmons, P.J., Torok-Storb, B., "CD34 expression by stromal precursors in normal human adult bone marrow", 1991, Blood, 78, 2848-2853.
22. 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.
23. Köller, U., Peschel, CH., "Cluster report: CD33", 1989, Leucocyte Typing IV, White Cell Differentiation Antigens. W. Knapp, et al., Eds., Oxford University Press, 812-813.
24. 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.