IOTest®3
CD14-FITC / CD13-PE / CD45-ECD

REF A07722
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	CD14	CD13	CD45
Clone	RMO52	SJ1D1	J33
Hybrid	SP2/0 x Balb/c	SP2/0 x Balb/c	NS1 x Balb/c
Immunogen	Isolated human monocytes	KG-1 cell line	Laz 221 cell line
Immunoglobulin	IgG2a	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 CD14, CD13 and CD45 expression 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 leucocytes is performed by incubating the sample with the IOTest 3 reagent. Red cells are then removed by lysis and the leucocytes, which are unaffected by this process, are analysed by flow cytometry.
The flow cytometer analyses light diffusion and the fluorescence of cells. It makes possible the localisation of cells within an electronic window defined on a histogram, which correlates the orthogonal diffusion of light (Side Scatter or SS) with the fluorescence of the ECD, corresponding to CD45 staining. Other histograms combining two of the different parameters available on the cytometer are also used in the gating stage.
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 CD45 antigen, on a Side Scatter representation *versus* CD45 expression to gate blast cells is helpful in the phenotyping of leukaemias (1 – 3). A slight to average expression of the CD45 antigen is characteristic of blast cells of myeloid origin (cases of acute myeloid leukaemia, AML) whereas no to weak expression is observed in blast cells of lymphoid origin (cases of acute lymphoblastic leukaemia, ALL) (1).
Simultaneous analysis of the expression of CD14 and CD13 antigens is useful for the differentiation of AMLs with a monocytic ontogeny ($CD14^+CD13^+$ phenotype) from AMLs with a non-monocytic ontogeny ($CD14^-CD13^+$ phenotype) (1 – 4).

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 homogenised by gentle agitation prior to taking the test sample.
Samples should be analysed within 24 hours of venipuncture.

METHODOLOGY

NECESSARY MATERIAL NOT SUPPLIED

- Sampling tubes and material necessary for taking samples.
- 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).
  - The following IOTest 3 negative control: 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 analysed, in addition to the test tube, one control tube is required in which the cells are mixed with the chosen 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 (i.e. the equivalent of approximately 5 x 10^5 cells). 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 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 analysed within 2 hours.

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

PERFORMANCE

SPECIFICITY

The CD14 molecule is a protein with a molecular weight of 53 – 55 kDa anchored in the membrane by means of a glycosyl-phosphatidylinositol group (GPI) (5).
The CD14 antigen is strongly expressed on monocytes and macrophages and moderately so on peripheral blood polynuclear neutrophils; it is also present on pleural phagocytes and dendritic reticular cells. CD14 is found on cells of the myelo-monocytic line and is only very weakly expressed by B lymphocytes. It is absent from T lymphocytes as well as from NK cells, erythrocytes and platelets (6, 7).
The monoclonal antibody (mAb) RMO52 was assigned to CD14 during the 6th HLDA Workshop on Human Leucocyte Differentiation Antigens, held in Kobe, Japan, in 1996 (WS Code: MA62, Section M).

The CD13 molecule, also known by the name of Aminopeptidase N (APN), is a type II transmembranous metalloprotease with a molecular weight of 150 – 170 kDa. This molecule has an extensive extracellular sequence and a short intracytoplasmic region. The CD13 molecule appears to bind as a homodimer in a non-covalent manner at the cell surface (8).
The CD13 antigen is expressed early on in the granulomonocytic cell line (monocytes, neutrophils, eosinophils and basophils) and their committed progenitors (intermediate progenitors forming mixed granulomonocyte colonies or CFU-GM) (8 – 11). The CD13 molecule is also expressed on cells from non-haematopoietic tissues such as epithelial cells, proximal renal tubules and cells making up the brush border of the intestine, endothelial cells, fibroblasts, stromal cells of bone marrow, osteoclasts and biliary tract basal cells (12).
MAb SJ1D1 reacts in peripheral blood with monocytes, neutrophils, eosinophils and basophils. SJ1D1 was assigned to the CD13 molecule during the 3rd HLDA Workshop in Oxford, England, in 1986 (WS Code: 285, Section M) (9).

The CD45 “molecule” is a concept which covers a series of isoform molecules identifiable by at least 4 specific antibody groups: CD45RA, CD45RB, CD45RC and CD45RO. These isoforms stem from the alternative splicing of 3 exons of a single gene coding for peptides A, B or C of the CD45 molecule (13). The CD45 family of glycoproteins, expressed on the surface of all human leucocytes, is absent from erythrocytes (14). The density of expression of the CD45 antigen on lymphocytes is greater than that seen on monocytes, which itself is greater than that seen on neutrophils (15).
MAb J33 reacts with all the isoforms of CD45 (the molecular weight of which varies between 180 to 220 kDa): it is therefore referenced as a pan-leucocyte marker. MAb J33 was assigned to the CD45 molecule during the 3rd HLDA Workshop in Oxford, England, in 1986 (WS Code: 818, Section NL) (16).

LINEARITY

To test the linearity of this reagent, two cell lines THP1 ($CD14^+CD13^+CD45^+$) and FRN14.33 ($CD14^-CD13^-CD45^-$) were mixed in different proportions with a constant cell quantity so that the THP1 / FRN14.33 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)
CD14	Y = 0.98 X + 0.99	0.999
CD13	Y = 0.97 X + 1.38	0.999
CD45	Y = 1.00 X + 0.03	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, samples of the whole blood of 10 healthy adults were tested using the reagent described above. The mean values of the results obtained in the leucocyte sub-populations of interest in these 10 donors are shown in the tables below:

Lymphocytes	Number	Mean (%)	SD	CV (%)
$CD45^+$	10	96.1	5.5	5.7

Monocytes	Number	Mean (%)	SD	CV (%)
$CD14^+$	10	90.3	8.3	9.2
$CD13^+$	10	92.9	6.8	7.4
$CD45^+$	10	90.3	8.3	9.2

Granulocytes	Number	Mean (%)	SD	CV (%)
$CD14^+$	10	99.8	0.1	0.1
$CD13^+$	10	99.6	0.4	0.4
$CD45^+$	10	99.8	0.1	0.1

INTRA-LABORATORY REPRODUCIBILITY

On the same day and using the same cytometer, 12 measurements of the percentage of positive cells were performed on a target population expressing these three markers (IMMUNO-TROL™ Control Cells Ref. 6607077). The results obtained are shown in the following table:

Monocytes IMMUNO-TROL	Number	Mean (%)	SD	CV (%)
$CD14^+$	12	71.6	1.2	1.7
$CD13^+$	12	99.8	0.1	0.1
$CD45^+$	12	99.8	0.1	0.1

INTER-LABORATORY REPRODUCIBILITY

On the same day and on the same population of monocytes expressing these three markers (IMMUNO-TROL Control Cells), 12 measurements of the percentage of positive cells were performed by two technicians and the preparations analysed using two different cytometers. The results obtained are shown in the following tables:

Cytometer n°1:

Monocytes IMMUNO-TROL	Number	Mean (%)	SD	CV (%)
$CD14^+$	12	71.6	1.2	1.7
$CD13^+$	12	99.8	0.1	0.1
$CD45^+$	12	99.8	0.1	0.1

Cytometer n°2:

Monocytes IMMUNO-TROL	Number	Mean (%)	SD	CV (%)
$CD14^+$	12	75.1	1.4	1.8
$CD13^+$	12	99.9	0.1	0.1
$CD45^+$	12	99.9	0.1	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 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

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

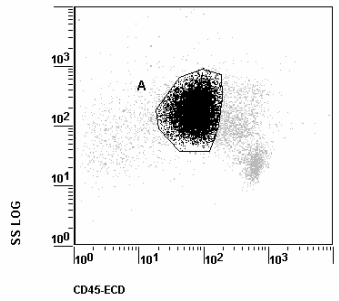
www.beckmancoulter.com

APPENDIX TO REF A07722

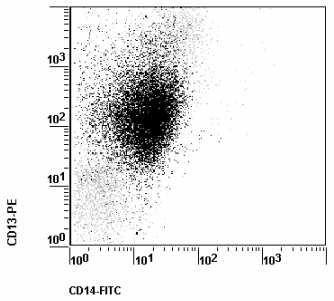
EXAMPLES

The 4 diagrams below are biparametric representations (Side Scatter *versus* Fluorescence Intensity or Fluorescence Intensity *versus* Fluorescence Intensity) of one specimen stained with IOTest 3 CD14-FITC / CD13-PE / CD45-ECD Conjugated Antibodies (Ref. A07722). Lysis and fixation are with IOTest 3 Lysing Solution (Ref. A07799) and IOTest 3 Fixative Solution (Ref. A07800) respectively. All acquired events are represented. 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).

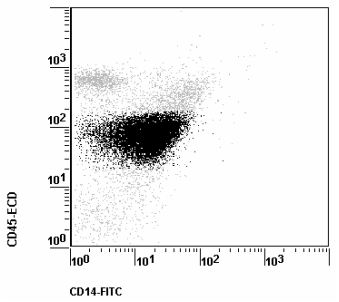
Analyzed case: M3 Acute Myeloblastic Leukemia
Bone marrow sample. Region A defines the gating strategy (CD45$^{dim\ to\ positive}$ cluster) used on this example.



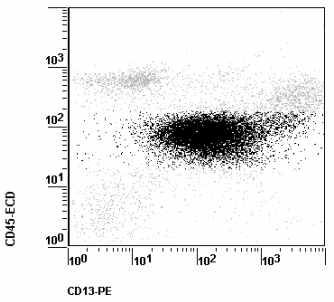
Histogram 1



Histogram 2



Histogram 3



Histogram 4

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. 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.
5. Goyert, S.M., Cohen, L., Gangloff, S.C., Ashmun, R., Haeffner-Cavaillon, N., "CD14 Workshop panel report", 1997, Leucocyte Typing VI, White Cell Differentiation Antigens. Kishimoto, T., et al, Eds., Garland Publishing, Inc., 963-965.
6. Todd III, R.F., Nadler, L.M., Schlossman, S.F., "Antigens on human monocytes identified by monoclonal antibodies", 1981, J. Immunol., 126, 1435-1442.
7. Todd, R.F., van Agthoven, A., Schlossmann, S.F., Terhorst, C., "Structural analysis of differentiation antigens Mo1 and Mo2 on human monocytes", 1982, Hybridoma, 1, 329-337.
8. Goyert, S.M., "CD 13 workshop panel report", 1997, Leucocyte Typing VI, White Cell Differentiation Antigens. Kishimoto, T., et al, Eds., Garland Publishing, Inc., 962-963.
9. Hogg, N., Horton, M.A., "Myeloid antigens, new and previously defined cluster", 1987, Leucocyte Typing III, White Cell Differentiation Antigens, McMichael A.J., et al., Eds., Oxford University Press, 576-602.
10. Pierelli, L., Teopili, L., Menichella, G., Rumi, C., Paolini, A., Iovino, S., Puggioni, P.L., Leone, G., Bizzi, B., "Further investigations on the expression of HLA-DR, CD33 and CD13 surface antigens in purified bone marrow and peripheral blood CD34$^+$ haematopoietic progenitor cells", 1992, Br. J. Haematol., 83, 1-7.
11. Mirro, J., Melvin, S., Metzger, D., Look, A., Murphy, S., "Changes in cell surface antigen expression during myelocytic and monocytic cell differentiation", 1984, Leucocyte Typing I, Bernard, A. et al. Eds., Springer Verlag, 442-446.
12. Ashmun, R.A., Holmes, K.V., Shapiro, L.H., Favalaro, E.J., Razak, K., de Crom, R.P.G., Howard, C.J., Look, A.T., "CD13 (aminopeptidase N) cluster workshop report", Leucocyte Typing V, White Cell Differentiation Antigens. Schlossman, S.F., et al., Eds., Oxford University Press, 771-775.
13. Weiss, L.M., Arber, D.A., Chang, K.L., "CD45: A review", 1993, Appl. Immunohistochem., 1, 166-181.
14. Sewell, W.A., Cooley, M.A., Hegen, M., "CD45 Workshop Panel Report", 1997, Leucocyte Typing VI, White Cell Differentiation Antigens. Kishimoto, T., et al, Eds., Garland Publishing, Inc., 499-502.
15. Poppema, S., Lai, R., Visser, L., Yan, X.J., "CD45 (Leucocyte Common Antigen) expression in T and B lymphocyte subsets", 1996, Leuk. Lymphoma, 20, 217-222.
16. 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.