IOTest®

CD14+16-FITC / CD85k-PE / CD33-PC5

REF A23413
50 tests; 1 mL
20 µL / test

BECKMAN COULTER

IOTest
Conjugated Antibodies

IVD CE

ENGLISH	Specification of constituent 1	Specification of constituent 2	Specification of constituent 3
Specificity	CD14+16	CD85k (ILT3)	CD33
Clone	RMO52+3G8	ZM3.8	D3HL60.251
Hybridoma	SP2/0 x Balb/c	Ag8.653 x Balb/c	NS1 x Balb/c
Immunogen	Human monocytes+ neutrophils	Fusion protein of human ILT3	HL60 human cell line
Immunoglobulin	IgG2a + 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)	Phycoerythrin (PE)	Phycoerythrin Cyanin 5.1 (PC5)
λ excitation	488 nm	488 nm	488 nm
Emission peak	525 nm	575 nm	670 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 identification and enumeration of the myeloid subset of circulating dendritic cells (MDC) in human biological samples.

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 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 (see examples in the Appendix for the description of the proposed gating strategy).

EXAMPLES OF CLINICAL APPLICATIONS

The simultaneous analysis of CD14, CD16, CD85k (ILT3) and CD33 antigens, helps the identification and the characterization of peripheral blood myeloid dendritic cells (MDCs), based on the selection of all myeloid cells with CD33 and the positive selection of MDCs + monocytes with ILT3. The discrimination of MDCs from monocyte subsets is then performed with CD14 + CD16.
Recent studies point to a numerical decrease and sometimes even functional impairment of circulating plasmacytoid and/or myeloid dendritic cells subsets in various pathologies.
Studies have shown a decrease in the number of circulating DCs in chronic Hepatitis B (1) or C patients (2) compared to non chronic and control individuals.
In haematopoietic cancer patients, DCs counts may also be significantly reduced in lymphoid or myeloid leukaemia (3 - 4). A similar observation is found in certain solid cancers (5). Furthermore, circulating DCs are decreased in metastatic cancers as compared to patients with localized cancer (6).

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 open 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 ingest and avoid 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.
Note: optimal results are obtained when the processed samples are analyzed within 6 hours after immunostaining.

METHODOLOGY

NECESSARY MATERIAL NOT SUPPLIED

- Sampling tubes and material necessary for sampling.
- Automatic pipettes with disposable tips for 10, 20, 100 and 500 µL.
- Plastic haemolysis tubes.
- Calibration beads. For example: Flow-Set™ Fluorospheres (Ref. 6607007).
- Red cell lysis reagent with washing stage after lysis. For example: VersaLyse™ (Ref. A09777).
- Leucocyte fixation reagent. For example : IOTest 3 Fixative Solution (Ref. A07800).
- Isotypic controls: IOTest reagents : IgG1-PC5 (Ref. A07798) and IgG1-FITC/IgG1-PE (Ref. A07794).
- Buffer (PBS: 0.01 M sodium phosphate; 0.145 M sodium chloride; pH 7.2).
- Centrifuge.
- Automatic agitator (Vortex type).
- Flow cytometer.

PROCEDURE

NOTE: The procedure below is valid for standard applications. Sample and/or VersaLyse volumes for certain Beckman Coulter applications may be different. If such is the case, follow the instructions on the application's technical leaflet.
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 the isotypic controls.

1. Add 20 µL of specific IOTest conjugated antibodies to each test tube, and 20 µL of IgG1-FITC/IgG1-PE and 10 µL of IgG1-PC5 to the 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 following the recommendations of the lysis reagent used.
   As an example, if you wish to use VersaLyse (Ref. A09777), refer to the leaflet and follow preferably the procedure called "with concomitant fixation", which consists of adding 1 mL of the "Fix-and-Lyse" mixture prepared extemporaneously. Vortex immediately for one second 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 150 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 PBS plus 0.1% of formaldehyde if the preparations are to be kept for more than 2 hours and less than **6 hours**. (A 0.1% formaldehyde PBS can be obtained by diluting 12.5 µL of the IOTest 3 Fixative Solution (Ref. A07800) at its 10X concentration in 1 mL of PBS).
   - 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.

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PERFORMANCE

SPECIFICITY

The CD14 molecule is found on cells of myelomonocytic lineage. It is strongly expressed on monocytes, macrophages, and weakly on neutrophils (7, 8). It is also present on pleural phagocytic cells and on reticular dendritic cells, on Langerhans cells, and histiocytes (9, 10). The monoclonal antibody (mAb) RMO52 does not react with T or B lymphocytes (7, 8).
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) (11).

The CD16 antigen is the low-affinity receptor for IgG (FcgammaRIII). The CD16 antigen exists in two different forms encoded by two different genes: FcgammaRIIIA (or III-2) and FcgammaRIIIB (or III-1). One is a transmembrane form (FcgammaRIIIA) expressed on NK cells, monocytes and macrophages. The other is a glycosyl-phosphatidylinositol (GPI)-anchored form (FcgammaRIIIB) only expressed on neutrophils (12, 13).
MAb 3G8 binds to the FcgammaRIIIA as well as (strongly) to the FcgammaRIIIB.
MAb 3G8 was assigned to CD16 during the 4th HLDA Workshop of Vienna, Austria, in 1989 (WS Code: 409, Section NL) (14).

CD85k, alias ILT3, is a member of a family of genes located on human chromosome 19. The ILT gene-derived proteins are the Immunologulin (Ig)-Like Transcripts (ILT), also known as Leucocyte Ig-like Receptors (LIR) and Monocyte / Macrophage Ig-like Receptors (MIR) (15 – 17). ILT / LIR / MIR are preferentially expressed on monocytes, macrophages, dendritic cells and granulocytes.
CD85k, otherwise known as ILT3, LIR-5, HM18, is a transmembrane protein of 60 kDa, with 2 extracellular Ig-SF domains, and 3 cytoplasmic ITIMs. The molecule is constitutively phosphorylated. It is selectively expressed by monocytes, macrophages and DCs (18).
MAb ZM3.8 is specific for human ILT3, it immunoprecipitates a protein of 60 kDa from monocytes, and is expressed in human blood samples by a subset of dendritic cells (18 - 20).

The CD33 antigen is a transmembrane monomeric glycoprotein with a molecular weight of 67 kDa. It is expressed by hematopoietic progenitor cells on colony-forming units for granulocytes, erythrocytes, monocytes and mega-karyocytes (CFU-GEMM) (21). It is also present on progenitors of granulocytes and mononuclear phagocytes (CFU-GM) and on early erythroid progenitors (BFU-E) (21).
MAb D3HL60.251 reacts with cells of myeloid origin, strongly on monocytes, and weakly on granulocytes of the peripheral blood. It does not react with mature lymphoid cells or lymphoid precursors.
MAb D3HL60.251 was assigned to CD33 at the 4th HLDA Workshop in Vienna, Austria, in 1989 (WS Code: 504, Section M) (22).

LINEARITY

To test the linearity of staining for the CD33 and CD85k specificities of this reagent, a positive THP1 cell line ($CD33^+$ $CD85k^+$) and a negative cell line MOLT4 ($CD33^-$ $CD85k^-$) were mixed in different proportions and a constant final number of cells, so that the positive cells/negative cell 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)
CD33	Y = 0.9641 X + 1.6763	0.998
CD85k	Y = 0.9595 X + 2.0434	0.997

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 31 healthy adults were used. The results obtained for the mean percentage of MDC amongst PBMC (peripheral blood mononuclear cells) are given in the table below:

MDC	n	Mean %	SD	CV (%)
$CD14^-/CD16^-$ $CD33^+$ $CD85k^+$	31	0.454	0.148	32.62

INTRA-LABORATORY REPRODUCIBILITY

On the same day and using the same cytometer, 12 measurements of the positivity of a sample containing positive cells (peripheral blood from the same donor) were carried out. The results obtained are summarized in the following table:

MDC	n	Mean %	SD	CV (%)
$CD14^-/CD16^-$ $CD33^+$ $CD85k^+$	12	0.567	0.031	5.388

INTER-LABORATORY REPRODUCIBILITY

On the same day and on the same sample containing positive cells (peripheral blood from the same donor), 12 measurements of the positivity 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:

MDC	n	Mean %	SD	CV (%)
$CD14^-/CD16^-$ $CD33^+$ $CD85k^+$	12	0.496	0.033	6.699

Cytometer n° 2:

MDC	n	Mean (%	SD	CV (%)
$CD14^-/CD16^-$ $CD33^+$ $CD85k^+$	12	0.499	0.038	7.686

LIMITATIONS OF THE TECHNIQUE

1. Flow cytometry may produce false results if the cytometer has not been aligned perfectly, if fluorescence spillover 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 antibody of this reagent is 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 specimen 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.

MISCELLANEOUS

See the Appendix for examples and references.

TRADEMARKS

MANUFACTURED BY:

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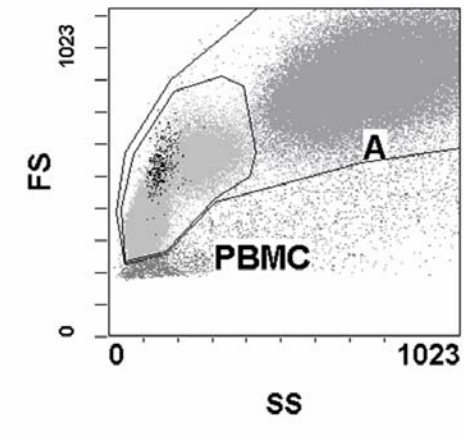
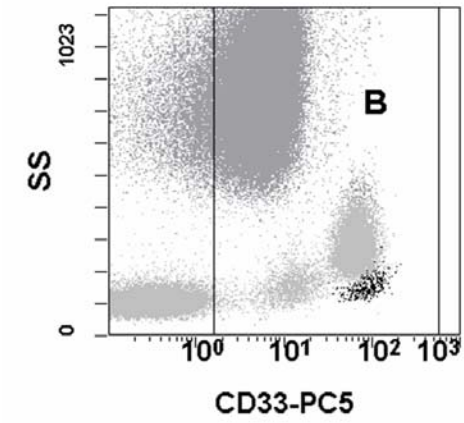
EXAMPLES

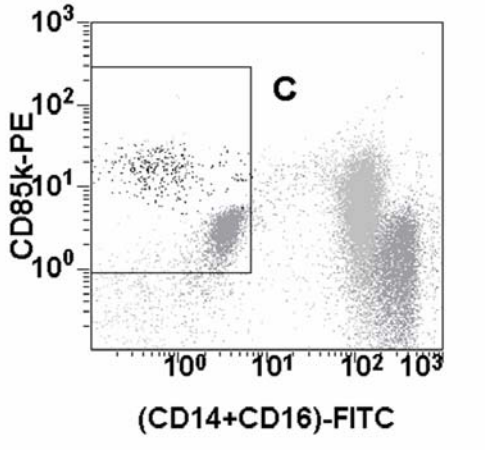
The histograms below are biparametric representations of a lyzed (see procedure described above) normal whole blood. The staining is performed with the 3-color combination :
(CD14+CD16)-FITC / CD85k(ILT3)-PE / CD33-PC5 (REF. A23413).



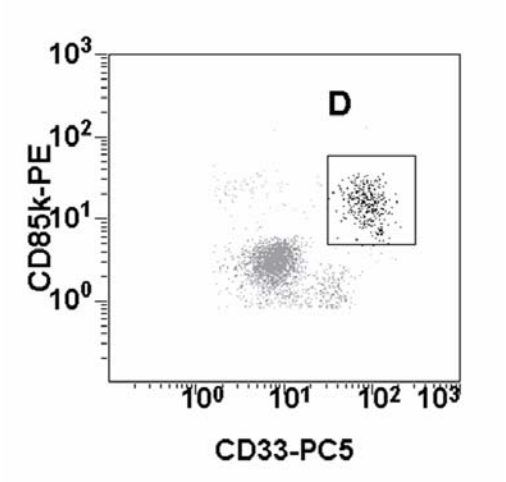
Histogram 1:
SS *versus* FS, display all events. Region A is drawn around white blood cells in order to eliminate debris. A Peripheral Blood Mononuclear Cell (PBMC) region including the lymphocytes and monocytes is drawn and the number of events counted in this region is used as denominator for the final calculation of the percentage of MDC.



Histogram 2 (gated on A):
FL4 (CD33-PC5) *versus* SS, display all events. Region B is drawn in order to exclude all CD33-negative cells.



Histogram 3 (gated on A AND B):
FL1 (CD(14+16)-FITC) *versus* FL2 (ILT3-PE).
Region C is drawn around all CD(14+16)$^{dim\ to\ neg}$/ ILT3^{+}.
As a visual control of the gating, the eosinophils (Eo) should be visible in the bottom right corner of the region C (see "Specificity").



Histogram 4 (gated on A AND B AND C):
FL4 (CD33-PC5) *versus* FL2 (ILT3-PE). Region D is drawn around CD33^{bright}/ILT3^{bright} events. (the percentage of MDCs amongst PBMC is calculated by dividing the number of events in gate D by the number of events in gate PBMC as defined on histogram 1).
Acquisition is with a COULTER® EPICS® XL™ flow cytometer, using System II™ acquisition sofwtare. Analysis is with Beckman Coulter CXP software.

REFERENCES

1. Duan, X. Z., Wang, M., Li, H. W., Zhuang, H., Xu, D., Wang, F. S., “Decreased Frequency and Function of Circulating Plasmacytoid Dendritic Cells (PDC) in Hepatitis B Virus Infected Humans”, J Clin Immunol. 2004 Nov, 24, 6, 637-646.
2. Tsubouchi, E., Akbar, S. M., Horiike, N., Onji, M., “Infection and dysfunction of circulating blood dendritic cells and their subsets in chronic hepatitis C virus infection”.J. Gastroenterol., 2004, 8, 754-762.
3. Mami, N. B., Mohty, M., Chambost, H., Gaugler, B., Olive, D., “Blood dendritic cells in patients with acute lymphoblastic leukaemia”, 2004, British Journal of Haematology, 126, 1, 77-80
4. Mohty, M., Isnardon, D., Vey, N., Brière, F., Blaise, D., Olive D., Gaugler, B., “Low blood dendritic cells in chronic myeloid leukaemia patients correlates with loss of $CD34^{+}/CD38^{-}$ primitive haematopoietic progenitors”, 2002, British Journal of Haematology, 119, 1, 115-118
5. Yanagimoto, H., Takai, S., Satoi, S., Toyokawa, H., Takahashi, K., Terakawa, N., Kwon, AH., Kamiyama, Y.,”Impaired function of circulating dendritic cells in patients with pancreatic cancer”, Clin Immunol. 2005; 114, 1, 52-60.
6. Lissoni, P., Vigore, L., Ferranti, R., Bukovec, R., Meregalli, S., Mandala, M., Barni, S., Tancini, G., Fumagalli, L., Giani, L., “Circulating dendritic cells in early and advanced cancer patients: diminished percent in the metastatic disease”,.1999, J. Biol. Regul. Homeost. Agents, 13, 216-219.
7. Todd III, R.F., Nadler, L.M., Schlossman, S.F., "Antigens on human monocytes identified by monoclonal antibodies", 1981, J. Immunol., 4, 126, 1435-1442.
8. Todd, R.F., van Agthoven, A., Schlossmann, S.F., Terhorst, C., "Structural analysis of differentiation antigens Mo1 and Mo2 on human monocytes", 1982, Hybridoma, 3, 1, 329-337.
9. Peters, J.H., Ruppert, J., Gieseler, R.K.H., Najar, H.M., Xu, H., "Differentiation of human monocytes into CD14 negative accessory cells: do dendritic cells derive from the monocytic lineage?", 1991, Pathobiology, 59, 122-126.
10. Ziegler-Heitbrock, H.W.L., Ulevitch, R.J., "CD14: Cell surface receptor and differentiation markers", 1993, Immunol. Today, 3, 14, 121-125.
11. 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.
12. Ravetch, J.V., Perussia, B., "Alternative membrane forms of FcgammaRIII (CD16) on human natural killer cells and neutrophils", 1989, J. Exp. Med., 170, 481-497.
13. Huizinga, T.W.J., Roos, D., Von dem Borne, A.E.G. Kr., "Neutrophil Fcgamma receptors: a two-way bridge in the immune system", 1990, Blood, 75, 1211-1214.
14. Schmidt, R.E., Perussia, B., "Cluster report: CD16", 1989, Leucocyte Typing IV, White Cell Differentiation Antigens. W. Knapp, et al., Eds., Oxford University Press, 574-578.
15. Samaridis, J., and Colonna, M., "Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways", 1997, Eur. J. Immunol., 3, 27, 660-665.
16. André, P., Biassoni, R., Colonna, M., Cosman, D., Lanier, L.L., Long, O., Lopez-Botet, M., Moretta, A., Moretta, L., Parham, P., Trowsdale, J., Vivier, E., Wagtmann, N., and Wilson, M.J., "New nomenclature for MHC receptors”, 2001, Nature Immunol., 2, 8, 661.
17. Marsh, S.G.E., Parham, P., Dupont, B., Geraghty, D.E., Trowsdale, J., Middleton, D., Vilches, C., Carrington, M, Witt, C., Guethlein, L.A., Shilling, H., Garcia, C.A., Hsu, K.C., and Wain, H., "Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002", 2003, Immunogenetics, 55, 220-226.
18. Cella, M., Döhring, C., Samaridis, J., Dessing, M., Brockaus, M., Lanzavecchia, A., Colonna, M., "A novel inhibitory receptor (ILT3) expressed on monocytes, macrophages, and dendritic cells involved in antigen processing", 1997, J. Exp. Med., 185, 1743-1751.
19. Mohty, M., Jarrossay, D., Lafage-Pochitaloff, M., Zandotti, C., Brière, F., de Lamballeri, X.N., Isnardon, D., Sainty, D., Olive, D., Gaugler, B., “Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment”, 2001, Blood, 13, 98, 3750-3756.
20. Chaperot, L., Bendriss, N., Manches, O., Gressin, R., Maynadie, M., Trimoreau, F., Orfeuvre, H., Corront, B., Feuillard, J., Sotto, J.J., Bensa, J.C., Brière, F., Plumas, J., Jacob, M.C., “Identification of a leukemic counterpart of the plasmacytoid dendritic cells”, 2001, Blood, 10, 97, 3210-3217.
21. Peiper, S.C., Andrews, R.G., "CD33 cluster workshop report", 1995, Leucocyte Typing V, 36 White Cell Differentiation Antigens Schlossman, S.F., et al., Eds., Oxford University Press, 837-840.
22. 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.