IOTest®3
cMPO-FITC / cCD79a-PE / cCD3-ECD

REF A07705
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	cMPO (intracellular MPO)	cCD79a (intracellular CD79a)	cCD3 (intracellular CD3)
Clone	CLB-MPO-1	HM47	UCHT1
Hybridoma	SP2/0 x CAF	NS1 x Balb/c	NS1 x Balb/c
Immunogen	Purified myeloperoxidase	Synthetic peptide (amino acids 202 – 216) from the cytoplasmic part of the CD79a (Mb-1) protein	Peripheral blood lymphocytes
Immunoglobulin	IgG1	IgG1, κ	IgG1, λ
Species	Mouse	Mouse	Mouse
Source	Ascites	Ascites	Ascites
Purification	Chromatography	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 intracellular expression of myeloperoxydase (MPO), CD79a and CD3 molecules in leucocytes.

PRINCIPLE

This test is based on the ability of specific monoclonal antibodies to bind to the antigenic determinants expressed by leucocytes on the internal surface of the plasma membrane, in the cytoplasm or in the nucleus.
Following a membrane fixation/permeabilization step, internal staining of the leucocytes is performed by incubating the sample with the IOTest 3 reagent. The leucocytes are then 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, from the 3 fluorescences present in the mixture, the one that corresponds to the most intense staining. The expression by the cell population thus gated of the other two markers is then analyzed 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 stained cells in relation to all the events acquired by the electronic gating.

EXAMPLES OF CLINICAL APPLICATIONS

The simultaneous analysis at the intracellular level, of MPO, CD79a and CD3 antigens, helps the identification and the characterization of acute hematological malignancies affecting the myeloid (MPO^+ $cCD79a^-$ $cCD3^-$), B lymphoid (MPO^- $cCD79a^+$ $cCD3^-$) and T lymphoid (MPO^- $cCD79a^-$ $cCD3^+$) cell lines (1). This reagent detects CD3 molecules on the surface as well as in the cytoplasm of stained cells.

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.
   Furthermore, 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 sample should be homogenized by gentle agitation prior to taking the test sample.
The samples must be analyzed within 24 hours of sampling.

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).
- Kits of compensation reagents : CYTO-COMP™ (Ref. 6607021 and 6607023).
- To obtain optimal results, the following reagents are recommended:
  - IntraPrep™ Fixation/Permeabilization reagent (Ref. A07802 or A07803).
  - Fixation reagent: IOTest 3 Fixative Solution (Ref. A07800).
  - The following IOTest 3 negative control: 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 the following procedure, the use of the IntraPrep Fixation/Permeabilization reagent (Ref. A07802 or A07803) is recommended.
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. A07732).
For a blood sample, optimal staining is obtained using a number of leucocytes between 3 and 10 x 10^3 cells / µL. If the leucocyte concentration is greater than 10 x 10^3 cells / µL, dilute. In the case of a cell suspension, optimal staining is obtained with 5 x 10^3 cells / µL; dilute if necessary.

1. Add 50 µL of blood sampled into EDTA or 5 x 10^5 cells to 2 tubes (one test tube, one control tube).
2. Add to each tube 100 µL of IntraPrep reagent 1 (Fixation).
3. Vortex the tubes vigorously for 3 to 5 seconds.
4. Incubate for 15 minutes at room temperature (18–25°C) protected from light.
5. Add 4 mL of PBS to each tube.
6. Centrifuge for 5 minutes at 300 x g at room temperature. Remove the supernatant by aspiration.
7. Add to each tube 100 µL of IntraPrep reagent 2 (Permeabilization). Let mix by diffusion. DO NOT VORTEX.
8. Incubate for 5 minutes at room temperature WITHOUT SHAKING.
9. Shake the tubes carefully and manually for 2 to 3 seconds.
10. Add 20 µL of the specific IOTest 3 conjugated antibody solution to the test tube. Add 20 µL of the negative control to the control tube. Vortex the tubes gently.
11. Incubate for 15 to 20 minutes at room temperature protected from light.
12. Add 4 mL of PBS and centrifuge at 300 x g for 5 minutes at room temperature.
13. Remove the supernatant by aspiration and resuspend the cell pellet in 0.5 to 1 mL of IOTest 3 Fixative Solution (Ref. A07800) at its working concentration (1X).
14. The preparations are ready for cytometric analysis.

NOTE: If the preparations are to be stored for more than 2 hours prior to cytometric analysis, it is advisable to store them at 2-8°C and protected from light. The preparations thus stored do not keep, however, for more than 24 hours.

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PERFORMANCE

SPECIFICITY

Myeloperoxydase (MPO) is an intracellular enzymatic protein expressed at an early stage in the differentiation of the myeloid cell line. MPO is synthesized primarily during the formation of promyelocytes, the stage during which azurophile granules (or primary granules) are formed (2 – 4). MPO is a major constituent of the azurophile granules of polynuclear neutrophils and macrophages, but is expressed neither by lymphocytes nor by erythrocytes (4 – 6).
The monoclonal antibody (mAb) CLB-MPO-1 recognizes the MPO as well as the MPO precursor (proMPO; the inactive form of the enzyme).

The CD79a molecule is part of the CD79a / CD79b disulphide-linked heterodimer, non-covalently bound to surface immunoglobulins to form B cell receptors (BCR) (7). The expression of CD79a appears early in the ontogeny of B cells and its localization at the pro-B stage is therefore cytoplasmic. Later on, the CD79a forms part of the BCR. Its membrane expression persists up to the plasmocytic stage, the stage at which its localization once again becomes cytoplasmic (8).
The mAb HM47 reacts with an intracytoplasmic epitope of the CD79a molecule (8). This antibody was assigned to CD79a at the 5th HLDA Workshop on Human Leucocyte Differentiation Antigens held in Boston, USA, in 1993 (WS Code: cB017, Section B) (8).

The CD3 antigen is a complex formed from 5 invariable polypeptide chains (γ, δ, ε, ζ and η), each with a molecular weight of between 20 and 25 kDa, and from the γ chain of receptors from the constant fractions (Fc) of immunoglobulins (FcγR). CD3 antigen is expressed on T lymphocytes and on a sub-population of thymocytes (9). In peripheral blood, approximately 67 to 76% of lymphocytes are $CD3^+$, this percentage, which varies according to age, is lower in young children (10).
The mAb UCHT1 recognizes the ε chain of CD3, both at the surface (11) as well as intracytoplasmically (cCD3) (12, 13). This antibody was assigned to CD3 at the first HLDA Workshop held in Paris, France, in 1982 (WS Code: 3, Section T) (14).

LINEARITY

To test the linearity of staining by this reagent, a pair of cell lines, one positive, the other negative, were selected for each marker. These cell lines 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. 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 (%)
cMPO	Y = 0.99 X + 0.26	0.999	1 — 98
cCD79a	Y = 0.99 X + 0.79	0.999	2 — 99
cCD3	Y = 0.99 X + 0.71	0.999	2 — 99

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 50 healthy adults were treated using the reagent described above. 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 (%)
$cCD79a^+$	50	11.9	5.3	44.2
$cCD3^+$	50	68.9	11.9	17.3

Granulocytes	Number	Mean (%)	SD	CV (%)
$cMPO^+$	50	99.3	1.1	1.2

INTRA-LABORATORY REPRODUCIBILITY

On the same day and using the same cytometer, 12 percentage measurements of leucocyte sub-populations were carried out on blood taken from the same donor. The results obtained are summarized in the following tables:

Lymphocytes	Number	Mean (%)	SD	CV (%)
$cCD79a^+$	12	19.6	1.1	5.8
$cCD3^+$	12	73	1.2	1.6

Granulocytes	Number	Mean (%)	SD	CV (%)
$cMPO^+$	12	99.7	0.1	0.1

INTER-LABORATORY REPRODUCIBILITY

On the same day and on blood from the same donor, 12 percentage measurements of leucocyte sub-populations 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:

Lymphocytes	Number	Mean (%)	SD	CV (%)
$cCD79a^+$	12	19.6	1.1	5.8
$cCD3^+$	12	73	1.2	1.6

Granulocytes	Number	Mean (%)	SD	CV (%)
$cMPO^+$	12	99.7	0.1	0.1

Cytometer n°2:

Lymphocytes	Number	Mean (%)	SD	CV (%)
$cCD79a^+$	12	18,4	0.5	2.9
$cCD3^+$	12	73.6	1.3	1.8

Granulocytes	Number	Mean (%)	SD	CV (%)
$cMPO^+$	12	98.5	0.7	0.7

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. 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.
3. The antibodies of this reagent are calibrated in order 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.
4. 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.
5. In certain disease states, such as severe renal failure or haemoglobinopathies, lysis of red blood 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:

IMMUNOTECH S.A.
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

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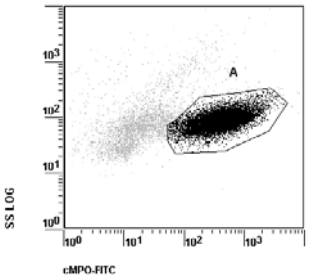
APPENDIX TO REF A07705

EXAMPLES

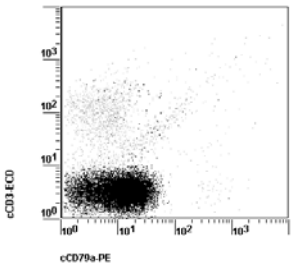
The 7 diagrams below are biparametric representations (Side Scatter *versus* Fluorescence Intensity or Fluorescence Intensity *versus* Fluorescence Intensity) of two specimens stained with IOTest 3 cMPO-FITC / cCD79a-PE / cCD3-ECD Conjugated Antibodies (Ref. A07705). Fixation and permeabilization are with IntraPrep Permeabilization Reagent (Ref. A07802 or Ref. A07803). 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).

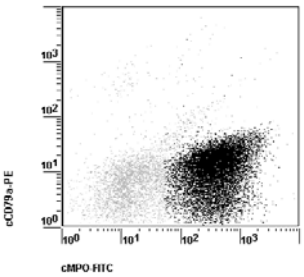
Case No. 1 (3 histograms): M1-type Acute Myeloid Leukemia (AML)
Bone marrow sample. Region A defines the gating strategy (MPO-positive cluster of blasts) used on this example.



Histogram 1

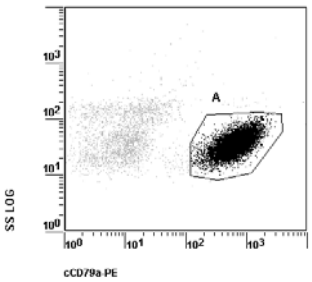


Histogram 2

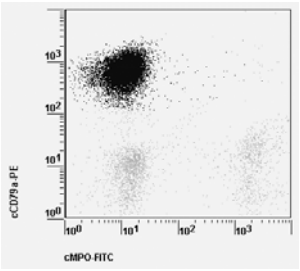


Histogram 3

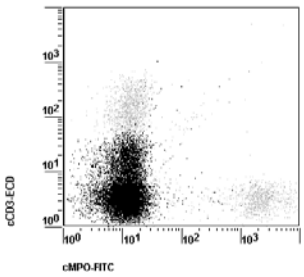
Case No. 2 (4 histograms): Acute B-Lymphocytic Leukemia (B-ALL)
Whole blood sample. Region A defines the gating strategy (CD79a-positive cluster of blasts) used on this example.



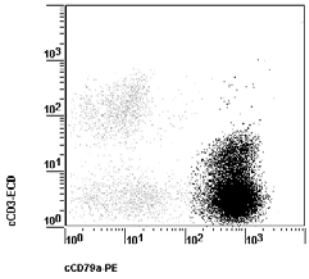
Histogram 4



Histogram 5



Histogram 6



Histogram 7

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