**IOTest®3**
**CD71-FITC / CD33-PE / CD45-ECD**

REF A07716
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** | CD71 | CD33 | CD45 |
| **Clone** | YDJ1.2.2 | D3HL60.251 | J33 |
| **Hybridoma** | X63-Ag8.8.653 x Balb/c | NS1 x Balb/c | NS1 x Balb/c |
| **Immunogen** | MLA 144 (leukaemic cell line of the gibbon) | 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 (E CD™) |
| **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 CD71, CD33, 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 light (Side Scatter) of analyzed cells with the ECD 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 CD71 and CD33 antigens is useful for distinguishing blast cells of myeloid origin ($CD71^-CD33^+$) from those having an erythroid origin ($CD71^+CD33^-$) (1, 2, 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.
   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 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:
  - Lysis 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 selected 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 total blood sampled in EDTA (or 5 x $10^5$ cells) in both 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

Antigen CD71, also known under the name of the T9 antigen and as the transferrin receptor, is a transmembrane glycoprotein. This molecule is expressed by precursors of the erythrocytic cell line, by reticulocytes and by brain endothelial cells (5). In general, CD71 is expressed by all cells undergoing proliferation, reflecting the demands for iron at this stage in the cell cycle (5).
The monoclonal antibody (mAb) YDJ1.2.2 was analyzed during the 5th HLDA Workshop on Human Leucocyte Differentiation Antigens, Boston, USA, in 1983 (WS Code: A006 and BP0111, Section AA6 and BP respectively) (6).

The CD33 antigen is a transmembrane monomeric glycoprotein with a molecular weight of 67 kDa. This molecule is part of the sialoadhesin group of compounds: its adhesive properties depend on the presence of sialic acid (7). 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 (8). The CD33 antigen is expressed strongly on monocytes and weakly on circulating granulocytes.
MAb D3HL60.251 was analyzed during the 4th HLDA Workshop in Vienna, Austria, in 1989 (WS Code: 504, Section M) (7).

The CD45 molecule includes at least 4 isoforms identifiable by the specific antibodies CD45RA, CD45RB, CD45RC and CD45R0. These isoforms of the CD45 molecule are the result of the alternative splicing of 3 exons from a single gene, coding for peptides A, B and C (9). The CD45 glycoprotein family is expressed on the surface of all human leucocytes, but is absent from erythrocytes (10). The density of expression of the CD45 antigen on lymphocytes is greater than that observed for monocytes and neutrophils (11).
MAb J33 reacts with all the isoforms of CD45 (180 to 220 kDa): it is therefore referenced as a pan-leucocytic marker. J33 was assigned to the CD45 molecule during the 3rd HLDA Workshop in Oxford, England, in 1986 (WS Code: 818, Section NL) (12).

### 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 ($R^2$) |
|---|---|---|
| CD71 | Y = 0.95 X + 1.56 | 0.999 |
| CD33 | Y = 0.99 X + 0.33 | 0.999 |
| CD45 | Y = 0.99 X + 0.33 | 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 10 healthy adults were treated 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. As no normal leucocyte population is positive for CD71, the expected values for this specificity are not given.

| Lymphocytes | Number | Mean (%) | SD | CV (%) |
|---|---|---|---|---|
| $CD45^+$ | 10 | 95.90 | 2.86 | 3 |

| Monocytes | Number | Mean (%) | SD | CV (%) |
|---|---|---|---|---|
| $CD45^+$ | 10 | 95.74 | 2.45 | 3 |
| $CD33^+$ | 10 | 91.71 | 4.92 | 5 |

| Granulocytes | Number | Mean (%) | SD | CV (%) |
|---|---|---|---|---|
| $CD45^+$ | 10 | 99.84 | 0.25 | 0.3 |
| $CD33^+$ | 10 | 99.52 | 0.47 | 0.5 |

### 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 cell line). The results obtained are summarized in the following table:

| MO7E line | Number | Mean (%) | SD | CV (%) |
|---|---|---|---|---|
| $CD71^+$ | 12 | 100.00 | 0.00 | 0.00 |
| $CD33^+$ | 12 | 90.67 | 0.40 | 0.44 |
| $CD45^+$ | 12 | 99.98 | 0.06 | 0.06 |

### INTER-LABORATORY REPRODUCIBILITY

On the same day and for the same target population (MO7E line), 12 measurements of the percentage of positive cells were carried out by two technicians and the preparations were analyzed using two different cytometers. The results obtained are summarized in the following tables:

Cytometer n°1:

| MO7E line | Number | Mean (%) | SD | CV (%) |
|---|---|---|---|---|
| $CD71^+$ | 12 | 100.00 | 0.00 | 0.00 |
| $CD33^+$ | 12 | 90.67 | 0,40 | 0.44 |
| $CD45^+$ | 12 | 99.98 | 0.06 | 0.06 |

Cytometer n°2:

| MO7E line | Number | Mean (%) | SD | CV (%) |
|---|---|---|---|---|
| $CD71^+$ | 12 | 100.00 | 0.00 | 0.00 |
| $CD33^+$ | 12 | 91.51 | 0.28 | 0.30 |
| $CD45^+$ | 12 | 99.81 | 0.03 | 0.03 |

## 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 lymphocyte origin of the blast cells should be confirmed using other markers.

## MISCELLANEOUS

See the Appendix for examples and references.

### TRADEMARKS



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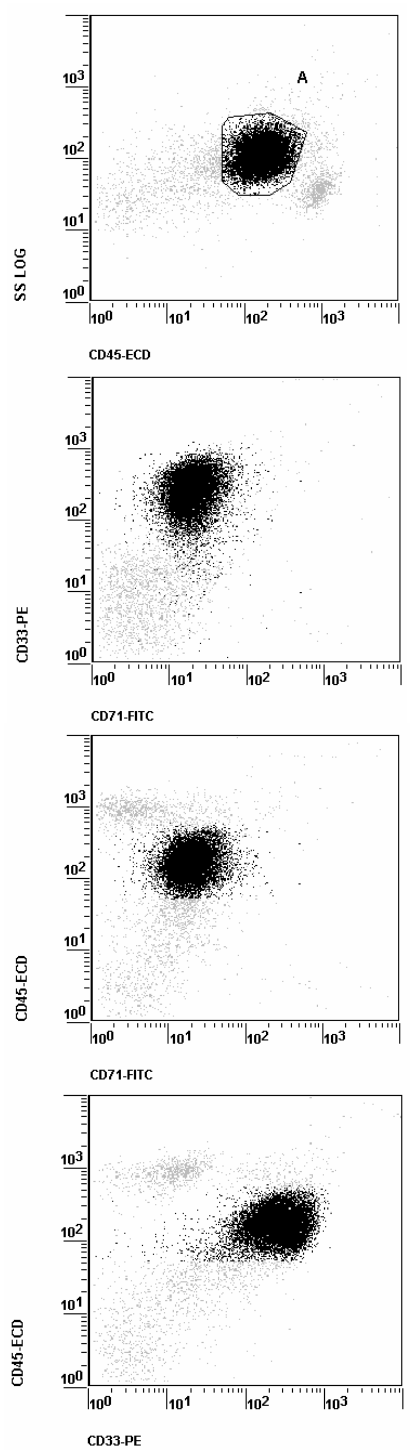
# APPENDIX TO REF A07716

## EXAMPLES

The 4 diagrams below are biparametric representations (Side Scatter versus Fluorescence Intensity or Fluorescence Intensity versus Fluorescence Intensity) of an Acute Myeloblastic Leukemia (bone marrow aspirate) (AML-M5) specimen. Staining is with CD71-FITC/CD33-PE/CD45-ECD Conjugated Antibodies (Ref. A07716). Lysis and fixation are with IOTest 3 Lysing Solution (Ref. A07799) and IOTest 3 Fixative Solution (Ref. A07800) respectively. All events acquired are shown. The blasts are shown in dark in all histograms. Region A defines the gating strategy (CD45 positive cluster) used on this example.

Acquisition is with a COULTER® EPICS® XL™ flow cytometer equipped with System II™ software. Analysis is with EXPO™ Cytometer software (Ref. 6605434).
Example: Acute Myeloblastic Leukemia (AML-M5).

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