IOTest®3
Neg. Ctrl.-FITC /
Neg. Ctrl.-PE /
CD3-ECD

REF A07731
25 tests; 0.5 mL
20 µL / test

IOTest 3
Conjugated antibodies

IVD

CE

ENGLISH	Specification of constituent 1	Specification of constituent 2	Specification of constituent 3
Specificity	Not applicable	Not applicable	CD3
Clone	679.1Mc7	679.1Mc7	UCHT1
Hybridoma	P3-X63-Ag.8.653 x Balb/c	P3-X63-Ag.8.653 x Balb/c	NS1 x Balb/c
Immunogen	Non-biological hapten	Non-biological hapten	Peripheral blood lymphocytes
Immunoglobulin	IgG1	IgG1	IgG1
Species	Mouse	Mouse	Mouse
Source	Ascites	Ascites	Ascites
Purification	Chromatography	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 (Negative Control-FITC / Negative Control-PE / CD3-ECD) is a negative control suitable for multiparametric analysis using flow cytometry as a support for gating. It makes possible the demonstration of the non-specific staining of all the IOTest 3s which use CD3-ECD.

PRINCIPLE

This test is based upon the ability of non-specific monoclonal antibodies to reproduce the non-specific staining of specific antibodies.
In parallel with the staining of leucocytes, which is performed by incubating the sample with a specific IOTest 3 reagent, another staining is also performed on the same sample with this negative IOTest 3 control. The erythrocytes of the two preparations are then removed by lysis and the leucocytes, 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 CD3 staining. Other histograms combining two of the different parameters available on the cytometer are also used in the gating stage.
The comparison of the levels of fluorescence obtained on the target population, on the one hand, with this negative control and, on the other hand, with the specific IOTest 3 staining, enables stained cells to be discriminated from non-stained cells (1, 2).

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.

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 specific conjugated antibody solutions from the IOTest 3 range which uses CD3-ECD as an aid to gating.
- 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 a control tube, a test tube is required in which the cells will be mixed with the specific IOTest 3 conjugated antibody solution.

1. Into each control tube, add 20 µL of the negative control and into each test tube, 20 µL of the specific IOTest 3 conjugated antibody solution.
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 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

The monoclonal antibody UCHT1 (mAb) was assigned to CD3 during the 1st HLDA Workshop on Human Leucocyte Differentiation Antigens, held in Paris, France, in 1982 (WS Code: 3, Section T) (3).
MAb 679.1Mc7 has structural characteristics identical to those of monoclonal antibodies specific to the IOTest 3 range but lacks any specificity vis a vis cellular antigens (1).

LINEARITY

To test the linearity of CD3 staining of this reagent, a positive cell line (HPBALL) and a negative cell line (RAMOS) were mixed in different proportions and 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)
CD3	Y = 0.997 X + 0.14	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. 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 (%)
$CD3^+$	50	72.9	9.1	13

INTRA-LABORATORY REPRODUCIBILITY

On the same day and using the same cytometer, 12 measurements of the percentage of staining of a positive target (HPBALL) were carried out. The results obtained are given in the following table as a percentage of stained cells for CD3 and as a Mean Fluorescence Intensity (MFI) for non-specific conjugated FITC and PE antibodies:

HPBALL	Number	Mean	SD	CV (%)
$CD3^+$ (%)	12	99.9	0.05	0.05
FITC (MFI)	12	1.09	0.03	3
PE (MFI)	12	0.11	0.001	0.7

INTER-LABORATORY REPRODUCIBILITY

On the same day and for the same positive target (HPBALL), 12 measurements of the percentage of stained cells were carried out by two technicians and the preparations analyzed using two different cytometers. The results obtained are given in the following tables as a percentage of stained cells for CD3 and as a Mean Fluorescence Intensity (MFI) for non-specific conjugated FITC and PE antibodies:

Cytometer n° 1:

HPBALL	Number	Mean	SD	CV (%)
$CD3^+$ (%)	12	99.9	0.05	0.05
FITC (MFI)	12	1.09	0.03	3
PE (MFI)	12	0.11	0.001	0.7

Cytometer n° 2:

HPBALL	Number	Mean	SD	CV (%)
$CD3^+$ (%)	12	99.9	0.04	0.04
FITC (MFI)	12	1.36	0.03	2.6
PE (MFI)	12	0.11	0.001	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. 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.

MISCELLANEOUS

See the Appendix for examples and references.

TRADEMARKS

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APPENDIX TO REF A07731

EXAMPLES

Conjugated Antibodies (Ref. A07731) Lysis and fixation are with IOTest 3 Lysing Solution (Ref. A07799) and IOTest 3 Fixative Solution (Ref. A07800) respectively.
Acquisition is performed with a COULTER® EPICS® XL™ flow cytometer equipped with System II™ software and analysis is with EXPO32™ ADC software.
The 2 histograms below are biparametric representations of a normal peripheral whole blood specimen.

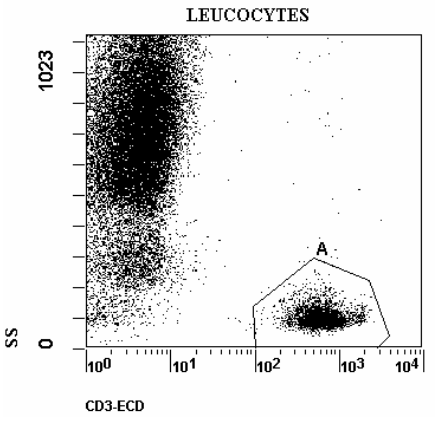

Figure 1:
Biparametric representation (Fluorescence Intensity *versus* Side Scatter) in order to gate on positive events (i.e. $CD3^+$).

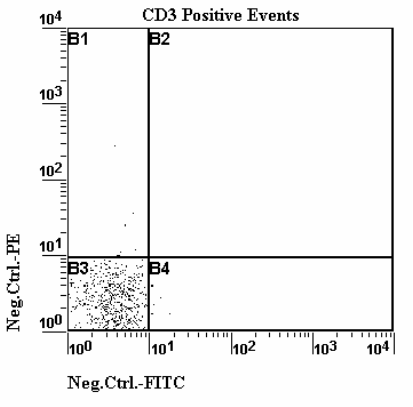

Figure 2:
Biparametric representation (Fluorescence Intensity versus Fluorescence Intensity) gated on $CD3^+$ events.

REFERENCES

1. Stewart, C.C., Stewart, S.J., "Cell preparation for the identification of leukocytes", 1994, Methods Cell Biol., Chap3, 41, 39-60.
2. Borowitz, M., Bauer, K.D., Duque, R.E., Horton, A.F., Marti, G., Muirhead, K.A., Peiper, S., Rickman, W., "Clinical applications of flow cytometry: Quality assurance and immunophenotyping of lymphocytes; approved guideline", 1998, NCCLS, 21, 18.
3. Bernard, A., Brottier, P., Georget, E., Lepage, V., Boumsell, L., "Joint report of the first International Workshop on Human Leucocyte Differentiation Antigens by the investigators of the participating laboratories", 1984, Leucocyte Typing I, Bernard, A. et al. Eds., Springer Verlag, 9-142.