

IOTest[®] Anti-Rat CD3-FITC / CD4-PC7 / CD8-APC

PN A32909 – Liquid 1.25 mL – 50 tests – 25 µL / test

For Research Use Only. Not for use in diagnostic procedures.

	Specifications of constituent 1	Specifications of constituent 2	Specifications of constituent 3
Specificity	CD3	CD4	CD8
Clone	1F4	OX-38	OX-8
Hybridoma	X63 myeloma x Balb/c	X63 myeloma x Balb/c	NS1 myeloma x Balb/c
Immunogen	F344 rat splenic T cells stimulated with PMA (TPA) and calcium ionophore	Rat thymocyte glycoproteins1	High molecular weight rat thymocyte membrane glycoproteins
Ig Isotype	IgM	IgG2a	IgG1
Species	Mouse	Mouse	Mouse
Source	Ascites fluid	Ascites fluid	Ascites fluid
Purification	Exclusion chromatography	Ion exchange or affinity chromatography	Ion exchange or affinity chromatography
Conjugation	Fluoresceine isothiocyanate (FITC)	R Phycoerythrin (PE) covalently linked to cyanine 7 (indotricarbocyanine)	Allophycocyanine (APC)
Molar Ratio	7 – 25 moles of FITC per mole of Ig	0.5 – 1.5 moles of PC7 per mole of Ig	0.5 – 1.5 moles of APC per mole of Ig.
Excitation wavelength	488 nm	488 nm	600 – 655 nm
Maximum emission wavelength	504 – 541 nm	750 – 810 nm	650 – 680 nm
Buffer	2 mg/mL bovine serum albumin in phosphate-buffered saline containing 0.1% sodium azide.		

INTENDED USE

Immunotoxicology studies that are performed on animals play a key role during the drug testing process and require reliable methods of determination of affected immune functions. Flow cytometry can be used to measure changes in specific lymphocytes enumerations and provides a useful assay to assess the immune status during pre-clinical assays (1, 2).

Cell immunophenotyping by flow cytometry using the multicolor IOTest Anti-Rat CD3-FITC / CD4-PC7 / CD8-APC reagent is the basis of an easy-to-use non-functional assay leading to rapid and reliable identification of specific cell populations that can be affected following drug action.

IOTest Anti-Rat CD3-FITC / CD4-PC7 / CD8-APC consists of an optimized three-color combination of fluorescent monoclonal antibodies that allows an accurate and robust characterization and enumeration of lymphocyte sub-populations (3-5). This multicolor combination is designed to discriminate and enumerate CD3⁺ T cell as well as CD4⁺ and CD8⁺ T cell sub-populations in Rats biological samples. It is designed to guarantee an easy method for multiparametric assays by flow cytometry analysis after red blood cell lysis using a No Wash procedure.

IOTest Anti-Rat CD3-FITC / CD4-PC7 / CD8-APC is intended for "For Research Use Only. Not for use in diagnostic procedures." and provides a straightforward tool to analyze a variety of biological samples such as whole blood, dissociated spleen cells or dissociated lymph node cells and bone marrow from Rats.

SPECIFICITY

Rat CD3:

The CD3 molecule is linked to the T cell receptor (TCR). It is expressed on mature T lymphocytes and on a sub-population of thymocytes.

The 1F4 mAb reacts with the CD3 antigen found on thymocytes, peripheral T lymphocytes, and dendritic epidermal T cells. It does not react with rat B cells or NK cells (6-8).

Rat CD4:

The CD4 antigen is a transmembrane molecule expressed by thymocytes and a sub-population of mature T cells. It is involved in the recognition of foreign antigens in association with the major histocompatibility complex (MHC) Class II antigens by T cells.

The OX-38 monoclonal antibody recognizes the rat CD4 antigen on most thymocytes, MHC Class II-restricted T cells (a sub-population of mature T lymphocytes including most T helper cells), monocytes, macrophages, and some dendritic cells (9-14).

Rat CD8:

The CD8 antigen is a disulfide-linked dimer formed by the association of two subunits (α and β) and acts as an accessory molecule to the TCR during T-cell activation restricted to the MHC Class I.

CD8 antigen is found on a subset of T lymphocytes and with a lower density, on a subset of NK cells and on thymocytes.

The monoclonal antibody OX-8 recognizes the α subunit of the CD8 heterodimer cell

surface antigen. It reacts with a subset of T lymphocytes, most thymocytes and the majority of NK cells, splenocytes, peripheral blood cells as well as lymph node cells of Rats (15-19).

TEST PRINCIPLE

The flow cytometer analyzes light diffusion and the fluorescence of cells. It makes possible the delimitation of the population of interest within successive electronic windows defined on histograms which correlate two of the different parameters available on the cytometer.

Histograms combining the orthogonal diffusion of light (Side Scatter or SS) and the diffusion of narrow-angle light (Forward Scatter or FS) are used as supports in the first gating stage for the application.

The fluorescence of the delimited cells is then analyzed in order to distinguish the positively-stained events from the unstained ones (20, 21).

The cell population of interest is stained with monoclonal antibodies. The red blood cells in each sample are then lysed prior to flow cytometry analysis using a No Wash procedure using VersaLyse™ Lysing Solution (PN IM3648) with the concomitant fixation using the IOtest3 Fixative Solution (PN IM3515) beforehand mixed so-called "Fix-and-Lyse" solution (a detailed procedure may be found in the VersaLyse package insert or in the paragraph below).

Once gating lymphocytes and monocytes, T lymphocytes (CD3- positive cells) are analyzed using a SS versus FL1 histogram. CD4⁺ T cells and CD8⁺ T cells are then analyzed using a FL5 versus FL4 histogram.

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APPLICATION

Flow cytometry

REAGENT

IOTest Conjugated Antibodies – Anti-Rat CD3-FITC / CD4-PC7 / CD8-APC
PN A32909 – 1 vial (1.25 mL, liquid format) – 50 tests – 25 µL / test.

PRECAUTIONS

1. This reagent contains 0.1% sodium azide. Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be flushed with running water while being discarded. These precautions are recommended to avoid deposits in metal piping in which explosive conditions can develop. If skin or eye contact occurs, wash excessively with water.
2. Specimens, samples and all material coming in contact with them should be handled as if capable of transmitting infection and disposed of with proper precautions.
3. Never pipet by mouth and avoid contact of samples with the skin mucosa and eyes.
4. Do not use antibody beyond the expiration date on the label.
5. Do not expose reagents to strong light during storage or incubation.
6. Avoid microbial contamination of reagents or incorrect results might occur.
7. Do not expose reagent to heat during storage or use.
8. Use general good laboratory practices when handling the reagents.

STORAGE CONDITIONS AND STABILITY

This reagent is stable up to the expiration date when stored at 2 – 8°C. Do not freeze. Minimize exposure to light. Once opened, the stability of the reagent is 90 days.

REAGENT PREPARATION

No reconstitution is necessary. This monoclonal antibody combination may be used directly from the vial. Bring reagent to 18 – 25°C prior to use.

PRECAUTIONS

Due to the tandem structure of the fluorochrome, PC7 also emits light at 575 nm. This secondary emission peak varies from lot-to-lot of PC7. Therefore, for multi-color analysis, the compensation matrix should be carefully checked when changing the lot of a PC7-conjugate.

SAMPLES

The specimen should be homogenized by gentle agitation prior sample pipetting and

should be used within 24 hours of harvesting. Samples must be analyzed within 2 hours.

INSTRUMENTS REQUIREMENTS

- The flow cytometer must be equipped to detect Forward Scatter, Side Scatter and three fluorescence channels allowing the analysis of FITC-, PC7-, and APC-conjugated antibodies (respectively 504 – 541 / 750 – 810 / 650 – 680 nm maximal peak emission).
- APC conjugates are usable only on flow cytometers equipped with an exciting source of 633 nm (He-Ne laser) or 635 nm (Red diode laser).
- Ensure that the flow cytometer is properly aligned for fluorescence intensity according to the manufacturer recommendations.

Example of photomultiplier (PMT) settings on Beckman Coulter instrument (FC500 flow cytometer equipped with MXP or CXP analysis software, version 2.0):

- While running Flow-Set Fluorospheres and Flow-Set 675 Fluorospheres from APC Set up Kit, adjust the PMT settings to place the peaks of scatter and fluorescence intensity distributions within the following target ranges.

Flow-Set Targets	
Parameter	Range
FS	116 – 132
FS675	35 – 50
SS	695 – 730
LOG FL1 (FITC)	13.2 – 16.1
LOG FL5 (PC7)	1.59 – 1.89
LOG FL4 (APC)	22.9 – 25.9

Refer to the instruction for use of Flow-Set Fluorospheres and APC Set-up for optimized standardization.

Example of procedure for fluorescence compensation:

- Identify 3 different tubes “CD45-FITC”, “CD45-PC7”, “CD45-APC”.
- Add 20 µL of IOTest CD45-FITC in tube “CD45-FITC”.
- Add 10 µL of IOTest CD45-PC7 in tube “CD45-PC7”.
- Add 10 µL of IOTest CD45-APC in tube “CD45-APC”.
- Add 100 µL of CYTO-TROL Control Cells in each tube.
- Vortex and incubate 20 minutes at room temperature. Then, add 0.5 mL of PBS 1X.

Refer to the instruction for use of CYTO-TROL Control Cells for optimized standardization.

METHODOLOGY

NECESSARY MATERIAL NOT SUPPLIED

- Deionized water
- Plastic test tubes (12 x 75 mm).

- Calibrated repeater pipet (20 µL, 100 µL, 1 mL) and tips
- PBS buffer (e.g. Beckman Coulter PN 6602489).
- Flow-Set™ Fluorospheres (Beckman Coulter PN 6607007)
- Flow-Check™ Fluorospheres (Beckman Coulter PN 6605359)
- APC (675/633) Set-up Kit (Beckman Coulter PN 6607120)
- IOTest CD45-FITC (Beckman Coulter PN IM0782)
- IOTest CD45-APC (Beckman Coulter PN IM2473)
- IOTest CD45-PC7 (Beckman Coulter PN IM3548)
- CYTO-TROL™ Control Cells Kit (Beckman Coulter PN 6604248)
- VersaLyse Lysing Solution (Beckman Coulter PN IM3648)
- IOTest 3 Fixative Solution (Beckman Coulter PN IM3515)
- Vortex mixer
- Centrifuge
- Timer
- Flow cytometer

SAMPLES PREPARATION

Suggested procedure as used in our laboratory to test the reagent on spleen cells or bone marrow:

- Harvest spleen or bone marrow cells in 40 mL of Hank's medium.
- Filtrate on Cell Strainer 100 µm.
- Perform two washes with Hank's by centrifugation for 5 minutes at 1200 rpm.
- Resuspend pellet into 5 mL of Hank's.
- Count and adjust cell suspension at 10x10⁶ cells / mL in Hank's.
- Proceed to staining according to paragraph “Procedure”.

Suggested procedure as used in our laboratory to test the reagent on lymph nodes:

- Harvest lymph nodes in 40 mL of Hank's medium.
- Dilacerate the lymph nodes.
- Filtrate on Cell Strainer 100 µm.
- Perform two washes with Hank's by centrifugation for 5 minutes at 1200 rpm.
- Resuspend pellet into 5 mL of Hank's.
- Count and adjust cell suspension at 10x10⁶ cells / mL in Hank's.
- Proceed to staining according to paragraph “Procedure”.

PROCEDURE

Preparation of working solutions (quantity for 1 tube):

- “Fix-and-lyse” mixture: by freshly mixing 1 mL of VersaLyse (PN IM3648) with 25 µL of undiluted IOTest 3 Fixative Solution (PN IM3515). Prepare a sufficient amount of the “Fix-and-lyse” mixture for the total number of samples.

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- Fixing buffer: by mixing 6.25 µL of undiluted IOTest 3 Fixative Solution (PN IM3515) in 0.5 mL PBS. Prepare a sufficient amount of the fixing buffer for the total number of samples.

NOTE: Unlike what is stated on the package insert of the IOTest 3 Fixative Solution (PN IM3515), the present procedure does not use this fixative solution as a 10X concentrated solution.

Specimen Requirements:

An absolute leukocyte count should be available for each specimen in order to be able to calculate the absolute counts of the lymphocyte sub-populations. The absolute count of leukocyte can be obtained from an automated cell counter or by manual counting of the nucleated cells. Alternatively, a flow cytometric method for absolute count determination, i. e. Flow-Count (PN 7547053), can be used in combination with the Versalyse "Fix and Lyse" no-wash procedure.

Procedure:

1. Label tubes for analysis.
2. Pipet into each tube 25 µL of the IOTest Anti-Rat CD3-FITC / CD4-PC7 / CD8-APC reagent.
3. Add 25 µL of whole blood.
4. Vortex each tube for 1 second.
5. Incubate at room temperature (18 – 25°C) for 20 minutes. Protect from light.
6. Add 1 mL of the "Fix-and-lyse" mixture to each tube and vortex immediately for one second after each addition.
7. Incubate at room temperature for at least 10 minutes. Protect from light.

Processed samples shall be stored at 2 – 8°C, protected from light, and analyzed within 2 hours.

EXAMPLE DATA

Histogram Creation:

Create histograms as follows:

- Create Histogram 1 as Forward Scatter vs Side Scatter.
- Create Histogram 2 as FL1 CD3-FITC vs Side Scatter.
- Create Histogram 3 as FL5 CD4-PC7 vs FL4 CD8-APC.

Gate Creation:

Create gates as follows:

1. Histogram 1 – Create a large Gate "A" to include all lymphocytes and monocytes while excluding debris.
2. Histogram 2 – Display events from Gate "A". Create Gate "B" to include CD3⁺ events (i.e. T lymphocytes).
3. Histogram 3 – Display events from Gate "B". Create Gate "C" to include CD8⁺ events and Gate "D" to include CD4⁺ events.

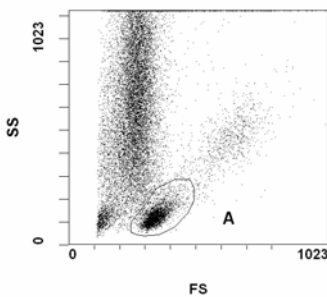
ANALYSIS EXAMPLE

The following histograms correspond to the analysis of the whole blood from one rat stained with the IOTest Anti-Rat CD3-FITC / CD4-PC7 / CD8-APC reagent.

NOTE: Below histograms were obtained on a BECKMAN COULTER CYTOMICS™ FC 500 flow cytometer equipped with the CYTOMICS CXP Software.

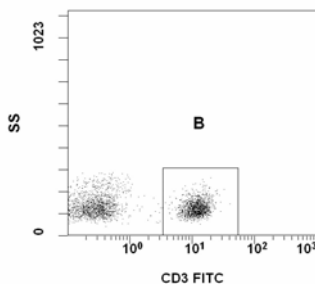
Histogram 1:

- Histogram 1 displays all events.
- Create a large Gate "A" to include all lymphocytes and monocytes while excluding debris.



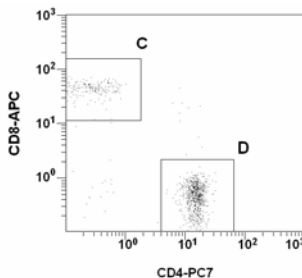
Histogram 2:

- Display events from Gate "A".
- Create Gate "B" to include CD3⁺ events (i.e. T lymphocytes).



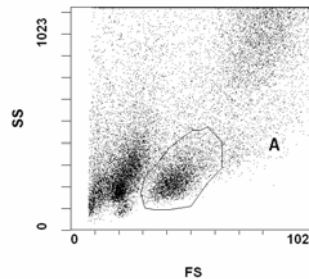
Histogram 3:

- Display events from Gate "B".
- Create Gate "C" to include CD8⁺ events and Gate "D" to include CD4⁺ events.

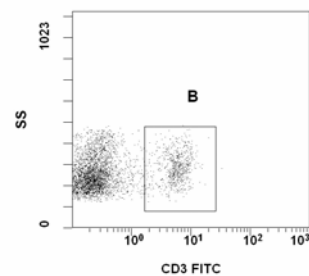


Example data concerning Rat bone marrow samples

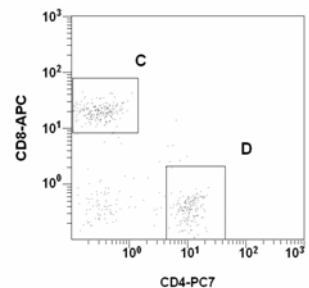
Histogram 1:



Histogram 2:



Histogram 3:

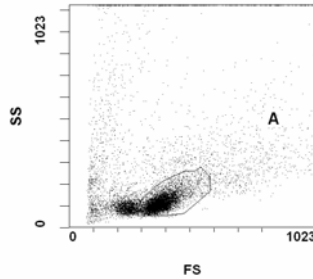


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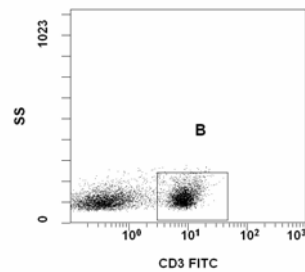
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Example data concerning Rat lymph node samples

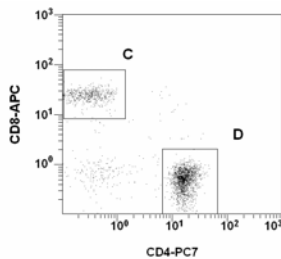
Histogram 1:



Histogram 2:

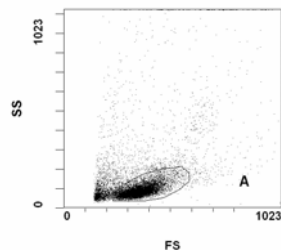


Histogram 3:

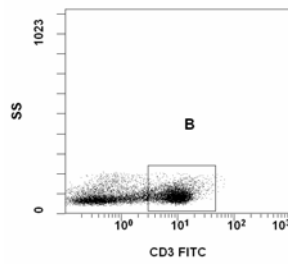


Example data concerning Rat spleen cell samples

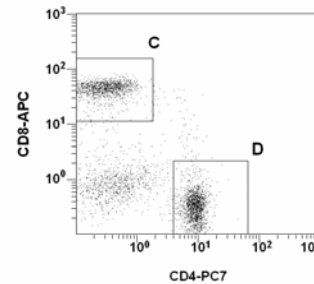
Histogram 1:



Histogram 2:



Histogram 3:



LIMITATIONS OF THE TECHNIQUE

1. Flow cytometry may produce false results if the cytometer has not been properly aligned, if fluorescence leaks have not been correctly compensated and if the gates have not been carefully positioned.
2. 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/number of cells ratio in every test.
3. Accurate and reproducible results will be obtained as long as the procedures used are in accordance with the technical package insert and compatible with good laboratory practices.
4. Visually verify the preparations to assess the efficacy of lysis. If they are cloudy or if the light diffraction histograms are unusual, it may be that lysis is incomplete.
5. The erythroblasts may be incompletely lysed and appear on a light diffraction histogram in the same location as the leucocytes.

LINEARITY

To test the linearity of staining of this reagent, different dilutions of whole blood from Wistar Rats were used. The counts were measured using comparable reagents from an alternative vendor. Aliquots were stained with the 3-color combination 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 = 1.012 X + 99.10	0.994
CD4	Y = 1.054 X - 931.28	0.983
CD8	Y = 1.096 X - 243.36	0.993

OBSERVED VALUES ON WISTAR RATS

Each laboratory shall compile a list of reference values based upon a group of healthy rats. This must be done by taking age, sex and strain group into account, as well as any other potential relevant differences.

In our laboratories, samples of the whole blood of 10 healthy Wistar Rats were treated using the reagent described above. The mean of percentage values of the results obtained in the lymphocytes sub-populations of interest in these 10 rats are shown in the tables below:

Lymphocytes	Nb	Mean (%)	SD
CD3 ⁺	10	43.67	8.35
CD4 ⁺	10	26.80	4.73
CD8 ⁺	10	17.68	3.81

INTRA-LABORATORY REPRODUCIBILITY

On the same day and on the same cytometer, 12 measurements of the percentage of staining of a positive target (lymphocytes from Wistar Rat) were carried out. The results obtained are summarized in the following table:

Lymphocytes	Nb	Mean (%)	SD	CV (%)
CD3 ⁺	12	49	1.02	2.09
CD3 ⁺ CD4 ⁺	12	61.26	0.76	1.23
CD3 ⁺ CD8 ⁺	12	36.21	0.73	2.03

INTER-LABORATORY REPRODUCIBILITY

On the same day and for the same population (lymphocytes from Wistar Rat), 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 summarized in the following tables.

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Cytometer n° 1:

Lymphocytes	Nb	Mean (%)	SD	CV (%)
CD3 ⁺	12	49	1.02	2.09
CD3 ⁺ CD4 ⁺	12	61.26	0.76	1.23
CD3 ⁺ CD8 ⁺	12	36.21	0.73	2.03

Cytometer n° 2:

Lymphocytes	Nb	Mean (%)	SD	CV (%)
CD3 ⁺	12	48.15	0.50	1.04
CD3 ⁺ CD4 ⁺	12	61.51	0.69	1.12
CD3 ⁺ CD8 ⁺	12	35.40	0.68	1.93

SELECTED RESEARCH REFERENCES

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PRODUCT AVAILABILITY

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PE is licensed under US patent 4,520,110

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