

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

### 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 single color IOTest Anti-Rat CD45-PE reagent is the basis of an easy-to-use non-functional assay leading to rapid and reliable identification of leucocytes. IOTest Anti-Rat CD45-PE consists of an optimized fluorescent monoclonal antibody designated for accurate and robust discrimination and enumeration of CD45-positive leukocytes in Rats biological samples. It enables the evaluation of a possible non-leucocytic contamination of the population of interest (3 – 5). It can be used with multicolor antibody combinations and therefore 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 CD45-PE is intended “*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.

### REAGENT

IOTest Anti-Rat CD45-PE Conjugated Antibody  
PN A36700 – 1 mL Liquid – 50 tests – 20 µL / test\*.

<b>Clone</b>	OX-1
<b>Isotype</b>	IgG1, mouse
<b>Immunogen</b>	Rat thymocyte 100kd glycoproteins fraction enriched for the rat Leukocyte Common Ag (LCA)
<b>Hybridoma Source</b>	NS1 x Balb/c spleen cells Ascites fluid
<b>Purification</b>	Ion exchange or affinity chromatography
<b>Conjugation</b>	R-phycoerythrin (PE) is conjugated at 0.5 – 1.5 moles of PE per mole of Ig.
<b>Fluorescence</b>	PE (orange-red) Excites at 486 – 580 nm Emits at 568 – 590 nm

### REAGENT CONTENTS

This reagent is provided in phosphate-buffered saline, with 0.1% sodium azide (NaN<sub>3</sub>) as preservative, and 2.0 mg / mL bovine serum albumin (BSA).

### SPECIFICITY

#### Rat CD45:

The OX-1 monoclonal antibody (mAb) recognizes rat Leucocyte Common Antigen (also called CD45) which is present on thymocytes, bone marrow cells, peripheral leucocytes but not on other tissues (6). CD45 is a major membrane glycoprotein which exists in different forms on different lymphoid cell types (7). It participates in lymphoid cell signal transduction during T-cell activation, as well as in intrathymic negative and positive selection (8).

### 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 (9, 10).

The cell population of interest is stained with monoclonal antibodies. The red blood cells are then lysed using a No Wash procedure. The recommended lysing reagent is the Fix-and-Lyse premix of VersaLyse™ Lysing Solution (PN IM3648) and IOTest3 Fixative Solution (PN IM3515) (a detailed procedure may be found in the VersaLyse package insert or in the paragraph below).

The anti-rat CD45-PE enables the evaluation of a possible non-leucocytic contamination of the lymphocytic gate during orthogonal light diffusion graphic analysis (Side Scatter) *versus* the fluorescence emitted by CD45 specific conjugated antibodies.

### APPLICATION

Flow cytometry

### 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 may be used directly from the vial. Bring reagent to 18 – 25°C prior to use.

### 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

Ensure that the flow cytometer is properly aligned for fluorescence intensity according to the manufacturer recommendations.

### 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).

# IOTest<sup>®</sup> Anti-Rat CD45-PE

PN A36700 – Liquid 1 mL – 50 tests – 20 µL / test

- 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  $10 \times 10^6$  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  $10 \times 10^6$  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.
- 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.

### Procedure:

1. Label tubes for analysis.
2. Pipet into each tube 20 µL of the IOTest Anti-Rat CD45-PE 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.

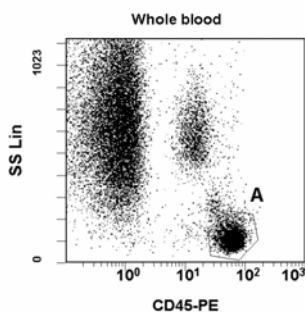
**Processed samples shall be stored at 2 – 8°C, protected from light, and analyzed within 2 hours.**

## EXAMPLE DATA

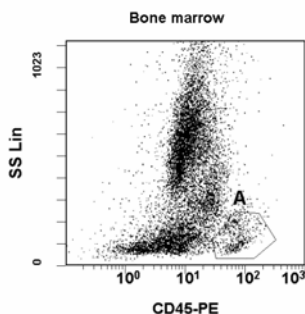
The following histograms correspond to the analysis of samples from Wistar rat stained with the IOTest Anti-Rat CD45-PE reagent.

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

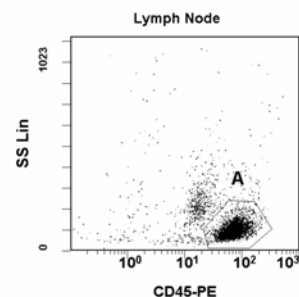
**Example data with Wistar Rat whole blood sample:**



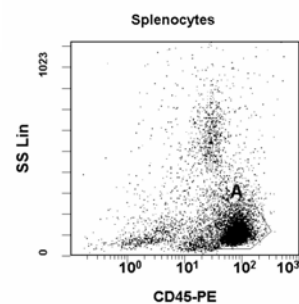
**Example data with Wistar Rat bone marrow samples:**



**Example data with Wistar Rat lymph node samples:**



**Example data with Wistar Rat spleen cells samples:**



## 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. This conjugated antibody is 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 on lymphocytes using comparable reagents from an alternative vendor.

Aliquots were stained with the reagent using the procedure described above and linear regression between the expected values and the observed values was calculated.

Specificity	Linear regression	Linearity (R <sup>2</sup> )
CD45	Y = 1.003 X + 51.80	0.999

### 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 leukocytes population of interest in these 10 rats are shown in the tables below:

Lymphocytes	Nb	Mean (%)	SD
CD45 <sup>+</sup>	10	98.48	1.04

### 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 (%)
CD45 <sup>+</sup>	12	96,91	1.34	1.39

### 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.

Cytometer n° 1:

Lymphocytes	Nb	Mean (%)	SD	CV (%)
CD45 <sup>+</sup>	12	96,91	1.34	1.39

Cytometer n° 2:

Lymphocytes	Nb	Mean (%)	SD	CV (%)
CD45 <sup>+</sup>	12	99.24	0.44	0.45

### SELECTED RESEARCH REFERENCES

1. Criswell, K. A., Bleavins, M. R., Zielinski, D., Zandee, J. C., "Comparison of flow cytometric and manual bone marrow differentials in Wistar rats", 1998, *Cytometry*, 32, 9 – 17.
2. Bollinger, A.P., "Cytologic evaluation of bone marrow in rats: indications, methods, and normal morphology", 2004, *Vet. Clin. Pathol.*, 33, 58 – 67.
3. Dean, J.H., House, R.V., Luster, M.I., 2001, "Immunotoxicology: Effects of, and Response to, Drugs and Chemicals", in *Principles and Methods of Toxicology*, Fourth Edition, Wallace Hayes, Ed., Taylor and Francis, Philadelphia, USA, 1415-1450.
4. Cooper-Hannan, R. et al., 1999, "The Principles of Good Laboratory Practices: Application to *In Vitro* Toxicology Studies. The Report and Recommendations of ECVAM Workshop 37", *ATLA*, 27, 539-577.
5. European Medicines Agency, 2006, ICH S8, Immunotoxicity studies for human pharmaceuticals. Note for guidance on Immunotoxicity studies for human pharmaceuticals. (EMEA/CHMP/167235/2004), 1 – 13.

6. Sunderland, C.A., McMaster, W.R., Williams, A.F., "Purification with monoclonal antibody of a predominant leukocyte-common antigen and glycoprotein from rat thymocytes", 1979, *Eur. J. Immunol.*, 9, 155-159.
7. Woollett, G.R., Barclay, A.N., Puklavec, M., Williams, A.F., "Molecular and antigenic heterogeneity of the rat leukocyte-common antigen from thymocytes and T and B lymphocytes", 1985, *Eur. J. Immunol.*, 15, 168-173.
8. Troiano, L., Monti, D., Cossarizza, A., Lovato, E., Tropea, F., Barbieri, D., Morale, M.C., Gallo, F., Marchetti, B., Franceschi, C., "Involvement of CD45 in dexamethasone and heat shock induced apoptosis of rat thymocytes", 1995, *Biochem. Biophys. Res. Com.*, 214, 941-948.
9. Dressler, L.G., "Specimen handling, storage, and preparation", 1997, *Curr. Protocols Cytometry*, Chapter 5, 1-5.
10. 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.

### PRODUCT AVAILABILITY

IOTest<sup>®</sup> Anti-Rat CD45-PE  
PN A36700 – Liquid 1 mL – 50 Tests – 20 µL / test

### TRADEMARKS

BECKMAN COULTER, the Beckman Coulter logo, CYTOMICS<sup>™</sup> FC 500, CYTOMICS<sup>™</sup> CXP Software, IOTest<sup>®</sup>, VersaLyse<sup>™</sup> are registered trademarks of Beckman Coulter, Inc.

PE is licensed under US patent 4,520,110