

Multicolor cocktails

CD3 – CD8- CD45- CD4

Fluorochrome	Reference	Test
FITC/PE/PerCP/APC	3F18PE145PP14A-50T	50 test



PRODUCT DESCRIPTION

Description: The anti-CD3/CD8/CD45/CD4 monoclonal antibody derives from human leukocytes (CD3), the hybridisation of mouse SP2 myeloma cells and spleen cells from BALB/c mice immunised with human T lymphocytes (CD8), T cells from leukemic HPB-ALL (CD45 and CD4).

Clone: 33-2A3, 143-44, D3/9, HP2/6

HLDA: CD3 → 2nd International Workshops on Human Leucocyte Differentiation

CD8 → 4th International Workshops on Human Leucocyte Differentiation, WS Code 169

CD45 → 6th International Workshops on Human Leucocyte Differentiation, WS Code N-L103

CD4 → 4th International Workshops on Human Leucocyte Differentiation, WS Code 116.

Isotype: Mouse IgG2a, IgG1, IgG1 and IgG2a kappa

Reactivity: Human

Source: Supernatant proceeding from an *in vitro* cell culture of a cell hybridoma.

Purification: Affinity chromatography.

Compositión: Mouse anti-human CD3/CD8/CD45/CD4 monoclonal antibody conjugated with a fluorochrome and in an aqueous solution which contains stabilising protein and 0.09% sodium azide (NaN₃).

Fluorochrome	Reagent provided	Concentration (µg/ml)
FITC (Fluorescein isothiocyanate)	25 µg in 2 ml	12,50
PE (R-Phycoerythrin)	5 µg in 2 ml	2,5
PerCP (Peridin-cholophyll-protein complex)	50 µg in 2 ml	25
APC (Allophycocyanin)	10 µg in 2 ml	5

RECOMMENDED USAGE

Immunostep's CD3/CD8/CD45/CD4, clones 33-2A3, 143-44, D3/9 and HP2/6 is a monoclonal antibody intended for *in vitro* diagnostic use in the identification and enumeration of human peripheral blood sample cells that express CD3, CD8, CD45 and CD4 on their surface using flow cytometry.

CLINICAL RELEVANCE

Suppressor/cytotoxic lymphocytes are a subset of T lymphocytes (CD3 +) that are CD8 +. Suppressor/ cytotoxic T-lymphocyte (CD3 + CD8 +) percentages may be used to characterize and monitor some forms of immunodeficiency and autoimmune diseases.

The percentage of suppressor/cytotoxic lymphocytes may lie outside the normal reference range in some autoimmune diseases, and in certain immune reactions such as acute graft-versus-host disease (GVHD) and transplant rejection.

The relative percentage of the CD8 + subset is elevated in many patients with either congenital or acquired immune deficiencies, such as severe combined immunodeficiency (SCID)⁽⁶⁻⁹⁾ and acquired immune deficiency syndrome (AIDS).

CD45 is a critical requirement for T and B cell antigen receptor-mediated activation and possible requirement for receptor-mediated activation in other leukocytes.

This reagent can be used in the characterization studies for immunophenotyping of leukocytes, which are widely applied in the characterization and follow-up of immunodeficiencies, autoimmune diseases, leukemias, etc. Detection of distinct isoforms can distinguish between naive T cells and memory T cells, which is of interest in patients with immunodeficiency and autoimmune diseases.

The identification of abnormal levels of CD4 + lymphocytes may be helpful in the diagnosis and / or prognosis of various immune diseases such as agammaglobulinemia, thymic aplasia (syndrome severe combined immunodeficiency and acquired immunodeficiency syndrome (AIDS).

Infection with human immunodeficiency virus (HIV), the causative agent of AIDS, resulting in profound immunosuppression due mainly to a selective reduction of CD4 + lymphocytes expressing the receptor for the virus (CD4 antigen). The progressive deterioration of clinical and immunological generally corresponds to a decrease in the count CD4 + lymphocytes.

PRINCIPLES OF THE TEST

The anti-CD3/CD8/CD45/CD4 monoclonal antibody binds to the surface of cells that express the CD3/CD8/CD45/CD4 antigens. To identify these cells, the sample is incubated with the antibody and is analysed by flow cytometry.

APPROPRIATE STORAGE AND HANDLING CONDITIONS

Store in the dark, refrigerated between 2 °C and 8 °C. DO NOT FREEZE. The antibody is stable until the expiry date stated on the vial label if kept at 2°C-8°C. Do not use after the date indicated.

Once the vial is open, the product is stable for 90 days.

EVIDENCE OF DETERIORATION

Reagents should not be used if any evidence of deterioration is observed. For more information, please contact our technical service: tech@immunostep.com

The product's normal appearance is a semi-transparent, colourless liquid. It should not be used if liquid medium is cloudy or contains precipitate. It should be odourless.

RECOMMENDATIONS AND WARNINGS

- a) The reagents contain sodium azide. In acid conditions, it is transformed into hydrazoic acid, a highly toxic compound. Azide compounds must be diluted in running water before being discarded. These conditions are recommended so as to avoid deposits in plumbing, where explosive conditions could develop. The safety data sheet (SDS) is available online at www.immunostep.com
- b) Avoid microbial contamination of the reagent.
- c) Protect from light. Use dim light during handling, incubation with cells and prior to analysis.
- d) Never mouth pipette.
- e) In the case of contact with skin, wash in plenty of water.
- f) The samples should be handled in the same way as those capable of transmitting infection. Appropriate handling procedures should be guaranteed.
- g) Do not use after the expiry date indicated on the vial.
- h) Deviations from the recommended procedure could invalidate the analysis results.
- i) For professional use only.
- j) Before acquiring the samples, it is necessary to make sure that the flow cytometer is calibrated and compensated.

SAMPLE COLLECTION

The extraction of venous blood samples should be carried out in blood collection tubes using the appropriate anticoagulant (EDTA or heparin)¹.

For optimum results, the sample should be processed during the six hours following the extraction. Samples which cannot be processed within the 48 hours following the extraction should be discarded.

MATERIALS REQUIRED BUT NOT PROVIDED

- Isotype controls:

Fluorochrome	Isotype Control	Immunostep reference
FITC	Mouse IgG2a	ICIGG2AF-100UG
PE	Mouse IgG1	ICIGG1PE-50UG
PerCP	Mouse IgG1	ICIGG1PP-100UG
APC	Mouse IgG2a	ICIGG2AA-50UG

- STEPCOUNT counting tubes (ref. 1399991218)
- RBCX10 lysis solution (ref. RBCX10-50ML)
- CD-Chex Plus (Streck) normal and CD4 Low
- Centrifuge
- Commonly used 12 x 75-mm flow cytometry assay tubes
- Micropipettes for dispensing volumes from 5 µl to 2 ml
- Blood collection tubes with anticoagulant.
- Phosphate buffered saline (PBS) with 0.09% sodium azide. It is recommendable to add 0.5%BSA
- Vacuum system
- Lysing solution
- Flow cytometer equipped with laser and appropriate fluorochrome filters
- Vortex Agitator

INSTRUCTIONS FOR USE

Diluting RBCX10 Lysis Solution:

1. Dilute the 10X RBCX10 concentrate 1:10 with room temperature (20–25 °C) deionized water.
2. The prepared solution is stable for 1 month when stored in a glass or high-density polyethylene (HDPE) container at room temperature.

Performing Quality Control: In accordance with guidelines, we recommend running two levels of liquid control material (process control).

Controls should be run at least once each day that patient testing is performed.

Use commercial controls providing established values for percent positive and absolute counts with each run to assess system performance. Immunostep recommends using CD-Chex Plus (Streck) normal and CD4 Low as process controls.

To perform quality control: Thoroughly mix the appropriate CD-Chex Plus (Streck) control, or equivalent process control. See the IFU for the control for detailed instructions.

1. Stain the control sample using Immunostep's CD3/CD8/CD45/CD4 antibody as described in the following section. The control sample should be processed like patient samples to monitor the ongoing performance of the entire analytic process.
2. Acquire the stained control sample on the flow cytometer.
3. Visually inspect the CD45 vs SSC dot plot. The lymphocyte population should appear as a bright, compact cluster with low SSC. Monocytes and granulocytes should also appear as distinct clusters. Do not proceed with analysis if populations are diffuse and there is little or no separation between clusters.
4. Verify that the results are within the values reported on the Assay Values sheet.

Staining the Cells:

Use care to protect the tubes from direct light. Perform the procedure at room temperature. See Precautions and Interfering Conditions.

1. For each patient sample, label a 12 × 75-mm tube with the sample identification number. For absolute counts, label an STEPCOUNT counting tube in place of the 12 × 75-mm tube. **Note:** Before use, verify that the STEPCOUNT bead pellet is intact and within the metal retainer at the bottom of the tube. If this is not the case, discard the STEPCOUNT counting tube and replace it with another.
2. Pipette 20 µL of Immunostep's CD3/CD8/CD45/CD4 antibody into the bottom of the tube. **Note:** If using an STEPCOUNT counting tube, pipette the reagent onto the side of the tube, just above the metal retainer, without touching the bead pellet.
3. Pipette 50 µL of well-mixed, anticoagulated whole blood into the bottom of the tube. **Note:** If using an STEPCOUNT counting tube, we recommend using the reverse pipetting technique to pipette the sample onto the side of the tube just above the metal retainer. Avoid smearing blood down the side of the tube. If whole blood remains on the side of the tube, it will not be stained with the reagent and can affect results.
4. Cap the tube and vortex gently to mix.
5. Incubate for 15–30 minutes in the dark at room temperature (20–25 °C).
6. Add 450 µL of 1X RBCX10 lysis solution to the tube.
7. Cap the tube and vortex gently to mix.
8. Incubate for 15–30 minutes in the dark at room temperature (20–25 °C). The sample is now ready to be analyzed on the flow cytometer. If samples will not be analyzed immediately after staining, store them in the dark at room temperature (20–25°C).

Acquiring the Samples:

1. Vortex the cells thoroughly at low speed. It is important to reduce aggregation before running samples on the flow cytometer. **Note:** If you are using a Loader, vortex tubes immediately before placing them into the Loader racks.
2. Install the tube on the cytometer and acquire the sample. Before acquiring samples, adjust the threshold to minimize debris and ensure that populations of interest are included.
3. Analyze the data using the appropriate cytometer-specific software. See the cytometer's IFU for more information.

Acquire on a flow cytometer or store in the dark at 2°C -8°C until the analysis is carried out. Samples should be acquired within the 3 hour after lysis.

FLOW CYTOMETRY ANALYSIS

Collect the fluorescence attributed to monoclonal antibody CD3/CD8/CD45/CD4 and determine the percentage of stained cells.

It is necessary to use an isotope control conjugated with the same fluorochrome, of the same type of immunoglobulin heavy chain and concentration as that of the CD3/CD8/CD45/CD4, so as to evaluate and correct the unspecific binding of lymphocytes (*please see materials required but not provided*). Set an analysis region to eliminate fluorescence background noise and to include positively stained cells. Below is an example diagram of peripheral blood stained applying the protocol described in point 6:

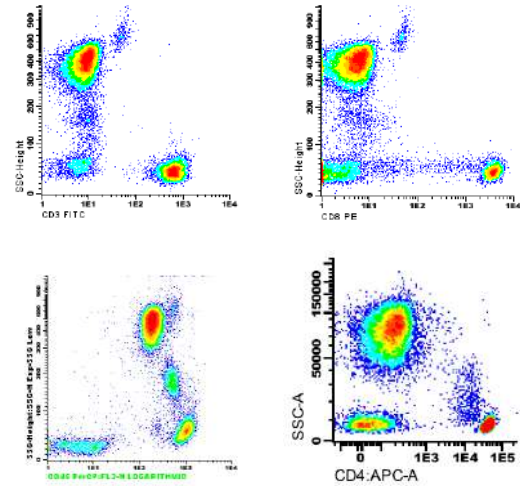


Fig. 1: A biparametric diagram of the average fluorescence intensity of the CD3 + ,CD8+, CD45+ and CD4+ leucocytes population and its internal complexity (SSC) in a peripheral blood specimen from a healthy donor.

Results: Results are reported as the percentage of positive cells per lymphocyte population or as the number of positive cells per microliter of blood (absolute count).

Calculating Absolute Counts: During analysis, the absolute number (cells/µL) of positive cells in the sample can be determined by comparing cellular events to bead events.

If Immunostep software is used, absolute counts will be determined by the software. For manual data analysis, the absolute count of the cell population (A) can be calculated using the following equation:

$$A=X/Y \times N/V$$

Where:

- X is the number of positive cell events
- Y is the number of bead events
- N is the number of beads per test, which is found on the STEPCOUNT counting tubes foil pouch and can vary from lot to lot
- V is the sample volume (50 µL)

Controls:

- Positive Controls: Use CD-Chex Plus (Streck) normal and CD4 Low to validate the staining protocol.
- Negative Controls: Include isotype controls and unstained cells to set proper gating strategies.

Material Not Supplied:

- STEPCOUNT counting tubes (ref. 1399991218)
- RBCX10 lysis solution (ref. RBCX10-50ML)
- CD-ChexPlus (Streck) normal and CD4 Low

LIMITATIONS OF THE PROCEDURE

1. Incubation of antibody with cells for other than the recommended procedures may result in a reduction or loss of antigenic determinants from the cell surface.
2. The values obtained from normal individuals may vary from laboratory to laboratory; it is therefore suggested that each laboratory should establish its own normal reference range.
3. Abnormal cells or cell lines may show a higher antigen density than normal cells. In some cases, this could require the use of a greater quantity of monoclonal antibody than is indicated in the procedures for sample preparation.
4. In whole blood samples, red blood cells found in abnormal samples, as well as nucleated red cells (from both normal and abnormal specimens) may be resistant to lysis. Longer periods of red blood cell lysing may be needed in order to avoid the inclusion of unlysed cells in the lymphocyte gated region.
5. Blood samples should not be refrigerated for an extensive period (more than 24 hours), since the number of viable cells will gradually decrease, and this may have an effect on the analysis. In order to obtain the best values, they should be kept at room temperature immediately prior to incubation with the monoclonal antibody.
6. Accurate results with flow cytometric procedures depend on correct alignment and calibration of the lasers, as well as correct gate settings.

REFERENCE VALUES

Abnormal results in the percentage of cells expressing the antigen or in its levels of expression may be due to pathological conditions. It is advisable to know the normal antigen expression patterns in order to ensure a proper interpretation of the results

The values obtained from healthy individuals may vary from laboratory to laboratory; it is therefore suggested that each laboratory should establish its own normal reference range.

CHARACTERISTICS

SPECIFICITY

Blood samples were obtained from healthy normal donors of Caucasian were stained with Immunostep CD3 FITC monoclonal antibody. Cells contained in the lymphocyte, monocyte and granulocyte regions were selected for analysis. Blood samples were processed by a leukocyte method, with a direct immunofluorescence staining for flow cytometric analysis.

To evaluate the reagent's Specificity (cross-reactivity with other cell populations), 10 blood samples from healthy donors were studied, stained with an adequate isotype control and the MAb to study. The percentage of lymphocytes, monocytes and granulocytes stained with the mentioned MAb was evaluated. The results obtained are shown in the following table:

Case summaries CD3+

	% lymphocytes	% monocytes	% granulocytes
1	64,99	1,10	,64
2	73,46	7,45	,56
3	51,29	7,00	,35
4	88,18	1,24	,71
5	70,27	12,70	,47
6	82,98	,21	,06
7	85,46	6,47	1,51
8	80,42	2,61	2,04
9	64,19	2,63	,50
10	64,72	7,10	,65
TOTAL	-	-	-
N	10	10	10
Mean	72,5960	4,8510	,7490
Median	71,8650	4,5500	,6000
Minimum	51,29	,21	,06
Maximum	88,18	12,70	2,04
Desv. Tip.	11,68110	3,93365	,58485
Variance	136,448	15,474	,342

Cases summaries (gate on Lymphocytes)

	% lymph. CD3+	% lymph. CD3+/CD4+	% lymph. CD3+/CD8+
1	64,99	58,89	22,25
2	73,46	39,57	24,72
3	51,29	30,97	14,73
4	88,18	71,68	14,86
5	70,27	55,82	34,51
6	82,98	62,22	26,28
7	85,46	70,40	18,85
8	80,42	37,74	20,60
9	64,19	50,88	36,54
10	64,72	62,15	13,09
TOTAL	-	-	-
N	10	10	10
Mean	72,5960	54,0320	22,6430
Median	71,8650	57,3550	21,4250
Minimum	51,29	30,97	13,09
Maximum	88,18	71,68	36,54
Desv. Tip.	11,68110	13,96613	8,05991
Variance	136,448	195,053	64,962

SENSIBILITY

Sensibility was assessed within a White Blood Cells (WBC) concentration of $0,2 \times 10^3$ to 20×10^3 WBC/ μ l and a lymphocyte concentration of $0,1 \times 10^3$ to $9,0 \times 10^3$ cells and a lymphocyte/ μ l concentration of 0.1×10^3 to 9×10^3 lymphocyte/ μ l. Results were observed to be linear within the CD3+ CD4 + range, the CD3 + CD8 + range, and the CD3 + range.

Sensitivity of the Immunostep CD3 FITC/ CD8 PE/ CD45 PerCP/ CD4 APC monoclonal antibodies was checked the concentration scale of stained cells obtained. The results show an excellent correlation level between the results obtained and expected based on the dilution used.

To determine the consistency of the conjugated monoclonal antibody as opposed to small variations (but deliberate). It provides an indication of its reliability during its normal use.

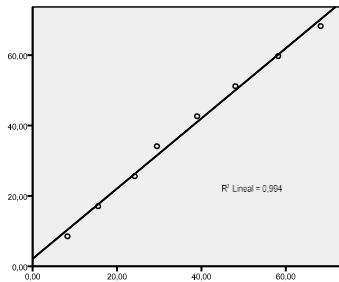
Case Summaries

	Sample	Dilution	% Expected	% Obtained
1	400A + 0B	100,0	68,26	68,26
2	350A + 50B	87,5	59,72	58,18
3	300A + 100B	75,0	51,18	48,06
4	250A + 150B	62,5	42,65	38,98
5	200A + 200B	50,0	34,12	29,46
6	150A + 250B	37,5	25,59	24,18
7	100A + 300B	25,0	17,06	15,51
8	50A + 350B	12,5	8,53	8,25
9	0A + 400B	,0	,00	,
TOTAL	-	-	-	-
N	9	9	9	8

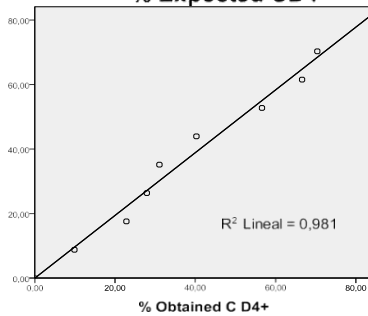
Model	R	R Square	Adjusted R Square	Std. Error of the estimate
1	,997(a)	,994	,993	1,177019

a Predictors: (Constant), Obtained

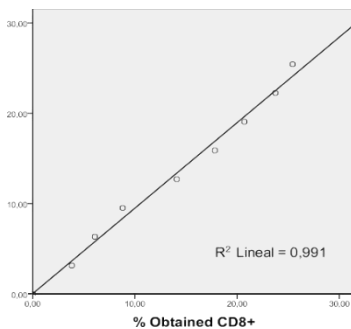
% Expected CD3+ cells



% Expected CD4+



% Expected CD8+ cells



REPRODUCIBILITY

Reproducibility for the Immunostep CD3 FITC/ CD8 PE and CD3 FITC/ CD4 APC-conjugated monoclonal antibodies was determined by performing 9 replicated determinations of each antibody in each of three lymphocyte ranges, high, medium and low. Thus, a total of 27 determinations were performed for each form of lymphocytes. In this manner, reproducibility was demonstrated throughout the entire measuring range. The 9 determinations for each range were performed by the staining, processing and analysis of 9 separate samples. Lymphocytes were selected for the analysis of percent cells stained in each of the three ranges.

To perform this study, anticoagulated blood was obtained from a normal donor expressing a high, medium and low percentage of lymphocytes. The study was performed in each of three independent laboratories, in the manner that each laboratory obtained, stained and analyzed separate blood samples.

Case Summaries CD3+

	% High	% Medium	% Low
1	69,64	67,02	56,00
2	69,78	63,14	56,57
3	69,56	64,64	59,17
4	58,45	63,03	52,86
5	68,73	63,69	56,52
6	69,51	64,85	52,94
7	69,47	64,92	52,63
8	68,48	65,40	49,33
9	69,38	63,58	52,00
TOTAL	-	-	-
N	9	9	9

Reproducibility CD3+

	% High	% Medium	% Low
N Valid	9	9	9
N Missing	0	0	0
Mean	68,1111	64,4744	54,2244
Median	69,4700	64,6400	52,9400
Moda	58,45*	63,03*	49,33*
Desv. Std.	3,64849	1,27407	3,02778
Variance	13,311	1,623	9,167
Range	11,33	3,99	9,84
Minimum	58,45	63,03	49,33
Maximum	69,78	67,02	59,17

Reproducibility CD3+/CD4+

	% High	% Medium	% Low
N Valid	9	9	9
N Missing	0	0	0
Mean	55,6089	59,1933	58,6244
Median	55,4400	59,4900	59,7900
Moda	53,77	54,74	54,61
Desv. Std.	1,28043	2,54627	2,26331
Variance	1,640	6,484	5,123
Range	3,35	7,43	5,87
Minimum	53,77	54,74	54,61
Maximum	57,12	62,17	60,48

Reproducibility CD3+/CD8+

	% High	% Medium	% Low
N Valid	9	9	8
N Missing	0	0	1
Mean	35,5878	33,0311	37,3213
Median	35,9600	33,3000	37,8750
Moda	33,83	25,48	40,00
Desv. Std.	1,25670	4,62543	3,66363
Variance	1,579	21,395	13,422
Range	3,49	12,27	9,57
Minimum	33,83	25,48	33,29
Maximum	37,32	37,75	42,86

WARRANTY

Warranted only to conform to the quantity and contents stated on the label or in the product labelling at the time of delivery to the customer. Immunostep disclaims hereby other warranties. Immunostep's sole liability is limited to either the replacement of the products or refund of the purchase price.

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