

# Anti-Human CD4 (HP2/6)

Fluorochrome	Reference	Test
FITC	4F-100T	100 test
PerCP	4PP-100T	100 test
APC	4A-100T	100 test



## PRODUCT DESCRIPTION

**Other Names:** T-cell surface glycoprotein CD4, T-cell surface antigen T4/Leu-3

**Description:** The anti-CD4 monoclonal antibody derives from the hybridisation of mouse SP2 myeloma cells and spleen cells from B/ALB/c mice immunised with human T lymphocytes. The antibody is formed by an IgG2a heavy chain and a kappa light chain.

**Clone:** HP2/6

**HLDA:** The anti-CD4 antibody, clone HP2/6, was included in the 4<sup>th</sup> International Workshops on Human Leucocyte Differentiation, WS Code 116.

**Isotype:** Mouse IgG2a, kappa

**Reactivity:** Human

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

**Purification:** Affinity chromatography.

**Composition:** Mouse anti-human CD4 monoclonal antibody conjugated with a fluorochrome and in an aqueous solution which contains stabilising protein and 0.09% sodium azide (NaN<sub>3</sub>).

Fluorochrome	Reagent provided	Concentration (µg/ml)
FITC (Fluorescein isothiocyanate)	50 ug in 2 ml	25
PerCP (Peridincholophyll-protein complex)	50 ug in 2 ml	25
APC (Allophycocyanin)	20 ug in 2 ml	10

## RECOMMENDED USAGE

Immunostep's CD4, clone HP2/6, is a monoclonal antibody intended for *in vitro* diagnostic use in the identification and enumeration of human sample lymphocytes that express CD4 using flow cytometry.

## CLINICAL RELEVANCE

The Immunostep CD4 monoclonal antibody may also be used, in combination with other indicators, for the diagnosis or prognosis of some immunodeficiency diseases, including Thymic differentiation and immune response.

CD4 is removed from plasma membrane by HIV-1 Nef protein that increases clathrin-dependent endocytosis of this antigen to target it to lysosomal degradation. Cell surface expression is also down modulated by HIV-1. Envelope glycoprotein gp160 that interacts with, and CD4 in the endoplasmic reticulum.<sup>1-5</sup>

## PRINCIPLES OF THE TEST

The anti-CD4 monoclonal antibody binds to the surface of cells that express the CD4 antigen. 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](mailto: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

- 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](http://www.immunostep.com)
- Avoid microbial contamination of the reagent.
- Protect from light. Use dim light during handling, incubation with cells and prior to analysis.
- Never mouth pipette.
- In the case of contact with skin, wash in plenty of water.
- The samples should be handled in the same way as those capable of transmitting infection. Appropriate handling procedures should be guaranteed.
- Do not use after the expiry date indicated on the vial.
- Deviations from the recommended procedure could invalidate the analysis results.
- FOR *IN VITRO* DIAGNOSTIC USE.
- For professional use only.
- 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)<sup>6,7</sup>. 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
PerCP		ICIGG2APP-100UG
APC		ICIGG2AA-50UG

- 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
- StepCount – 25 tests (Ref: 139999121). Reagent available for cell counting single platform, which can be used in combination with this reagent.
- Vacuum system
- Lysing solution
- Flow cytometer equipped with laser and appropriate fluorochrome filters
- Vortex Agitator

## SAMPLE PREPARATION:

The reagent is designed for protocols with and without washing after lysis. The latter is common in cell counting protocols.

### Lysis protocol with washing:

1. Add the suggested volume indicated on the antibody vial to a 12x75-mm cytometer tube. It is advisable to prepare an additional tube with the appropriate isotype control (please see materials required but not provided).
2. Add 100 µL of sample (up to 10<sup>6</sup> cells) and mix properly in the vortex.
3. Incubate in the dark for 15 minutes at room temperature (20-25°C) or for 30 minutes at 4°C.
4. Add 2 ml of the lysing solution, mix in the vortex and incubate in the dark for 10 minutes or until the sample is lysed.
5. Centrifuge at 540g for five minutes and carefully withdraw the supernatant by suction so as not to touch the cell pellet. Leave 5050 µl of non-aspirated liquid.
6. Resuspend pellet.
7. Add 2 ml of PBS (please see materials required but not provided).
8. Centrifuge 540g for five minutes and carefully withdraw the supernatant by suction so as not to touch the cell pellet. Leave 50 µl of non-aspirated liquid.
9. Resuspend the pellet in 0.3 ml of PBS.

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.

### Lysis protocol without washing:

1. Add the recommended volume of conjugated monoclonal antibody to a 12 x 75 mm flow cytometry tube. It is advisable to prepare an additional tube with the appropriate isotype control (see materials required but not supplied).
2. Add 50 µL of well-homogenised sample and mix thoroughly in the vortex.
3. Incubate in the dark at room temperature (20-25°C) for 15 minutes or at 4°C for 30 minutes.
4. Add 450 µl of lysis solution to the tube. Vortex and incubate in the dark for 10 minutes (or until the sample clarifies, indicating complete erythrocyte lysis).

The sample is now ready for analysis by flow cytometry.

### Technical recommendations:

- The lysis procedure without washing produces background (autofluorescence and unbound antibody signal) that can cause erroneous results. It is necessary to adjust the PMT voltages and compensation settings to optimise the method.
- It is critical to titrate the reagent to adjust it to the sample in order to minimise non-specific binding of unbound reagent, thus increasing the resolution between positive and negative populations.
- To obtain maximum data integrity, analyse samples within the validated stability period of the fluorochromes to avoid signal degradation or cell lysis artefacts.

## FLOW CYTOMETRY ANALYSIS

Collect the fluorescence attributed to monoclonal antibody CD4 and determine the percentage of stained T 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 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:

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. The use of commercial controls is also recommended, so Immunostep recommends using standard CD-Chex Plus (Streck) and CD4 Low as process controls. To perform quality control: thoroughly mix the appropriate CD-Chex Plus (Streck) control or an equivalent process control. Refer to the control's instructions for use for detailed instructions.

### CHARACTERISTICS

#### SPECIFICITY

The anti-CD4 antibody, clone HP2/6, was included in the Fourth Workshop on Human Leukocyte Differentiation Antigens (HLDA), using Code 116<sup>7</sup>. CD4 is present on 54% of peripheral blood T lymphocytes, 50% of thymocytes and some malignant cells of T cell origin. Normal B lymphocytes, monocytes or granulocytes do not express surface CD4 antigen although cytoplasmic expression has been observed in monocytes/macrophages. The CD4 positive T lymphocyte subpopulation has been characterised functionally as comprising helper cells active in amplification of immune responses.

Blood samples were obtained from healthy normal donors of Caucasian were stained with Immunostep CD4 PerCP mono-clonal antibody. Cells contained in the B lymphocyte, Platelets, Neutrophils granulocyte and Erythrocyte regions were selected for analysis. Blood samples were processed according Staining Cell Surface Antigens for Flow Cytometry Protocol.

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:

Statistics

		% Isotype control	% Platelets	% Erythrocytes	% B Lymphocyte	% Neutrophils
N	Valid	10	10	10	10	10
	Missing	0	0	0	0	0
Mean		0,50	0,02	0,24	0,01	0,17
Median		0,40	0,01	0,06	0,00	0,14
Std. Deviation		0,31	0,03	0,46	0,03	0,10
Minimum		0,01	0,00	0,00	0,00	0,08
Maximum		1,12	0,10	1,53	0,10	0,37
Percentiles	25	0,29	0,00	0,00	0,00	0,09
	50	0,40	0,01	0,06	0,00	0,14
	75	0,71	0,03	0,26	0,01	0,23

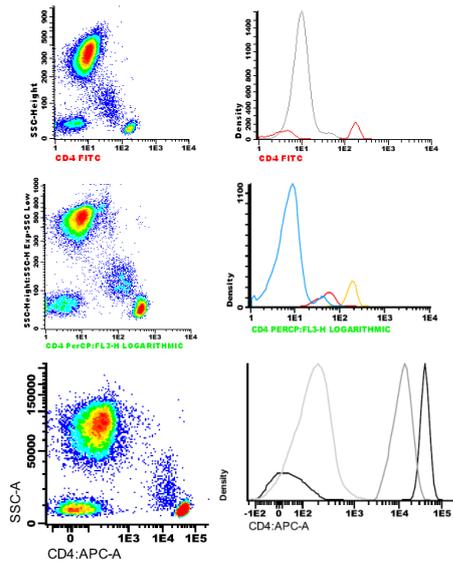


Fig. 1: On the left, a biparametric diagram of the average fluorescence intensity of the CD4+ T lymphocyte population and its internal complexity (SSC) in a peripheral blood specimen from a healthy donor. On the right, a diagram of the same specimen in histogram format.

### 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/ $\mu$ L) 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  $\mu$ L)

### 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.

## LINEARITY

Linearity of the Immunostep CD4 monoclonal antibody was determined by staining Raji cell line as positive population and Nalm-6 cell line as negative population (PerCP) and peripheral blood (FITC and APC). Cells were mixed in different proportions with a constant final number of  $1 \times 10^6$  cells to achieve different cell ratios from 0% positive cells to 100%.

Thereafter cells were incubated with the antibody according to the recommended amount for 15 minutes. Finally the cells were washed according to standard protocol. A linear regression between the expected values and the observed values was calculated.

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
FITC	0,995 <sup>a</sup>	0,99	0,98	0,43
APC	0,995 <sup>a</sup>	0,99	0,98	0,58
PerCP	0,994 <sup>a</sup>	0,98	0,98	4,07

## REPRODUCIBILITY

Reproducibility for the Immunostep CD4 conjugated monoclonal antibodies was determined by performing 10 replicated determinations of each antibody in each of three CD4+ ranges, high, medium and low. Thus, a total of 30 determinations were performed for each form of CD4. In this manner, reproducibility was demonstrated throughout the entire measuring range.

The 10 determinations for each range were performed by the staining, processing and analysis of 10 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 percentage of CD4+ cells. Mid-range and low range samples were obtained by mixing known CD4- cells in appropriate ratios, while maintaining the same total cell concentration for the three ranges.

The study was performed in each of three independent laboratories, in the manner that each laboratory obtained, stained and analyzed separate blood samples.

		N	Minimum	Maximum	Mean	Std. Deviation
APC	High	10	15,17	16,06	15,47	0,25
	Medium	10	2,94	3,29	3,14	0,11
	Low	10	1,00	1,44	1,29	0,12
	Valid N (listwise)	10				
FITC	High	10	1,93	2,14	2,03	0,06
	Medium	10	3,71	4,24	3,91	0,16
	Low	10	6,95	7,54	7,21	0,21
	Valid N (listwise)	10				
PerCP	High	10	15,17	16,06	15,47	0,25
	Medium	10	2,94	3,29	3,14	0,11
	Low	10	1,00	1,44	1,29	0,12
	Valid N (listwise)	10				

## 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.

## REFERENCES

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