

## Human Th1/Th2 Flow Cytometry Staining Kit

Cat. No: XJH001

Size: 20 Assays/100 Assays

### Kit Components

Cat.	Products	20 Assays	100 Assays	Storage
XJH001A	Human Th1/Th2 Cytokine Detection Antibody Cocktail	200 µL	1 mL	2~8°C, shading light
XJH001B	Human Th1/Th2 Cytokine Detection Antibody Isotype Cocktail	200 µL	1 mL	2~8°C, shading light
E-CK-A011	Cell Stimulation MIX Powder (50 µg)	50 µg	50 µg×3	-20°C/-80°C, shading light
E-CK-A012	Cell Stimulation MIX Solvent	120 µL	360 µL	~20°C, shading light
E-CK-A013	Protein Transport Inhibitor MIX Powder (200 µg)	200 µg	200 µg×3	-20°C/-80°C, shading light
E-CK-A109A	Fixation Buffer	10 mL	10 mL	2~8°C
E-CK-A109B	Permeabilization Buffer (5×)	15 mL	50 mL	2~8°C
	Manual		One Copy	

### Composition of Components

Products	Component
Human Th1/Th2 Cytokine Detection Antibody Cocktail	PerCP/Cyanine5.5 Anti-Human CD3 Antibody[OKT-3]
	Elab Fluor® 488 Anti-Human CD4 Antibody[SK3]
	APC Anti-Human IFN-γ Antibody[B27]
	PE Anti-Human IL-4 Antibody[MP4-25D2]
Human Th1/Th2 Cytokine Detection Antibody Isotype Cocktail	PerCP/Cyanine5.5 Anti-Human CD3 Antibody[OKT-3]
	Elab Fluor® 488 Anti-Human CD4 Antibody[SK3]
	APC Mouse IgG1, κ Isotype Control[MOPC-21]
	PE Rat IgG1, κ Isotype Control[HRPN]

Note: It is not recommended to mix Cocktail from different batches of kits.

### Storage

- The reagents in this kit can be stored for 12 months under the recommended conditions.
- Cell Stimulation MIX powder and Protein Transport Inhibitor MIX powder can be stored at -20°C and with shading light for 1 year, and at -80°C with shading light for 2 years. The dry powders can be dissolved and stored at -20°C and protected from light for 6 months, or can be dispensed and stored at -80°C and protected from light for 1 year.

### Introduction

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The Elabscience® Human Th1/Th2 Flow Cytometry Staining Kit can be used to detect the proportion of Th1 and Th2 cells among T lymphocytes (or CD4<sup>+</sup> helper T cells) in human anticoagulants. Th cells, also known as helper T cells (helper T cells), have a very weakly ability to differentiate from Th0 to Th1, Th2, and Th17 cells in the resting state (unstimulated state, e.g. normal physiological state in human). There are only a very small number of Th1, Th2 and Th17 cells in the peripheral blood, when the Th cells are stimulated by external factors (e.g. stimuli, pathogens, etc.), the Th0 will differentiate into Th1, Th2 or Th17, and the specific tendency of the differentiation will depend on the type of cytokine in the microenvironment. The proportion of Th1, Th2 or Th17 cells in the activated T lymphocytes can be known by detecting the expression of IFN- $\gamma$ , IL-4 or IL-17A in the sample. This product provides a mixture of four-color fluorescently labeled antibodies that specifically bind to human CD3, CD4, IFN- $\gamma$  (for detection of Th1), and IL-4 (for detection of Th2), allowing for the detection of Th1 and Th2 cells by multicolor panel.

Activated cells are stimulated to secrete cytokines by Cell Stimulation MIX in the kit, and the induced secretion of cytokines is further blocked intracellularly by the Protein Transport Inhibitor MIX. The activated T cells were fixed by Fixation Buffer and then permeabilized with Permeabilization Buffer to fully permeabilize the cell membrane, and the permeabilization could be synchronized with the binding of cell surface/intracellular antigens and antibodies, and finally the intracellular secretion of cytokines from the activated T cells was detected by flow cytometry. The surface markers of the Th cells are CD3<sup>+</sup>CD4<sup>+</sup>, and the correct gate was selected for CD4<sup>+</sup> T cells, and the proportion of Th1 and Th2 cells in the activated T lymphocytes was further analyzed by detecting different fluorescence signals on IFN- $\gamma$  and IL-4 antibodies.

## Materials Not Supplied

### ● Reagents

RPMI-1640, L-alanyl-L-glutamine solution (200 mM), penicillin-streptomycin solution, fetal bovine serum, cell staining buffer (E-CK-A107) or 1× PBS, Ficoll separation solution (1.077), DMSO, 10× ACK lysis buffer (E-CK-A105), deionized water.

### ● Instruments

Flow cytometer, CO<sub>2</sub> incubator, centrifuge.

## Reagent Preparation

### 1) 500× Cell Stimulation MIX

Add 100  $\mu$ L Cell Stimulation MIX Solvent to dissolve a vial of Cell Stimulation MIX Powder (50  $\mu$ g) and mix fully, and store at -20°C away from light.

**Note:** Centrifuge at 2000~10000×g for several seconds before use and then open the cover for use.

### 2) 1000× Protein Transport Inhibitor MIX

Add 50  $\mu$ L of pre-cooled 33% DMSO solution (supplied) to each tube of Protein Transport Inhibitor MIX Powder (200  $\mu$ g) and mix well to form 1000× Protein Transport Inhibitor MIX, store at -20°C away from light.

**Note:** Centrifuge at 2000~10000×g for several seconds before use and then open the cover for use. 33% DMSO solution can be prepared by mixing 670  $\mu$ L of sterile ultrapure water or sterile deionized water with 330  $\mu$ L of DMSO solution, and can be stored at -20°C away from light after preparation.

### 3) 1× Permeabilization Buffer

Dilute Permeabilization Buffer (5×) with deionized water to 1× Permeabilization Working Solution before use.

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For example, take 1 mL Permeabilization Buffer (5×) to 4 mL deionized water and mix fully to prepare 1× Permeabilization Working Solution.

Note: It is recommended that 1×Permeabilization Working Solution be used up within 3 days.

#### 4) RPMI-1640 complete medium

Add L-alanyl-L-glutamine solution (final concentration of 2 mM), penicillin solution (final concentration of 100 U/mL), streptomycin solution (final concentration of 100 U/mL), and fetal bovine serum (final concentration of 10%) to RPMI 1640 medium. The prepared medium can be stored at 4°C for two weeks.

## Experimental Protocol

### ➤ For human peripheral blood mononuclear cell (PBMC) sample

**Note: Sterile technique is required for the step 1-6.**

- Collect 10 mL of fresh human blood anticoagulated with a heparin sodium and proceed to the cell room for operations. The entire isolation process must be conducted under sterile conditions.  
Note: The PBMC separation operation should be carried out within 1 hour after collecting fresh human blood. Approximately  $1 \times 10^7$  PBMCs can be obtained from 10 mL of human blood. The Ficoll separation and centrifugation process should be carried out at  $20 \pm 2^\circ\text{C}$ . Higher temperatures may reduce cell yield, while lower temperatures may result in incomplete separation and reduced purity.
- Add 5 mL of Ficoll separation solution(1.077) (self-prepared) to two 15mL centrifuge tubes, respectively.. Then slowly add 5 mL of fresh human blood along the tube wall (mix the blood thoroughly before adding). Do not mix or shake the centrifuge tubes after adding the blood. Centrifuge at  $350 \times g$  for 20 min.
- Discard the top layer of supernatant (approximately 1~2 mL). Aspirate the second layer of supernatant and a small amount of the third layer of separation solution, and transfer it to a 50 mL centrifuge tube.
- Add 20 mL of RPMI-1640 basal medium (without serum) and mix fully. Centrifuge at  $250 \times g$  for 5 min, then discard the supernatant.  
Note: The cell pellet is generally grayish-white. If it appears red, it indicates the presence of residual erythrocyte. You can either use it directly or add an appropriate amount of red blood cell lysis buffer to lyse the red blood cells and obtain the target cells.
- Resuspend the cells in an appropriate amount of RPMI-1640 complete medium and count the cells. Adjust the cell density to  $1 \times 10^6$  cells/mL with the complete medium and seeded.
- Refer to the table below to add Cell Stimulation MIX and Protein Transport Inhibitor MIX. Incubate in a  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator for 5 hours, mix every 1~2 hours.

Group	Experimental Content	Addition of Antibodies
Negative group	Untreated	XJH001A
Test group	Add 2 μL of Cell Stimulation MIX (500×) and 1 μL of Protein Transport Inhibitor MIX (1000×) to each 1 mL cell suspension	
Isotype group		XJH001B

**In the subsequent steps, sterile technique is not necessary.**

- Collect the cells by centrifuging at  $300 \times g$  for 5 min. Discard the supernatant and resuspend the cells in 1 mL of Cell Staining Buffer or 1× PBS. Centrifuge again at  $300 \times g$  for 5 min and discard the supernatant.

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8. Each  $1 \times 10^6$  cells were resuspended with 180  $\mu$ L of Cell Staining Buffer or  $1 \times$  PBS, then add 60  $\mu$ L of Fixation Buffer and mix thoroughly. Fix the cells overnight at  $4^\circ\text{C}$  or at room temperature for 1 hour.
9. After fixation, the cells will settle at the bottom of the EP tube. Discard the supernatant and add 1~2 mL of  $1 \times$  Permeabilization Buffer. Mix fully and centrifuge at  $500 \times g$  for 5 min, and discard the supernatant.
10. Resuspend the cells in 100  $\mu$ L of  $1 \times$  Permeabilization Buffer. Add 10  $\mu$ L of the antibody to each group according to the table above. Incubate at room temperature with shading light for 60 min, mix every 15~20 min.
11. After incubation, add 1~2 mL of Cell Staining Buffer or  $1 \times$  PBS. Mix well and centrifuge at  $500 \times g$  for 5 minutes, and discard the supernatant.
12. Resuspend the cells in 100~200  $\mu$ L of Cell Staining Buffer or  $1 \times$  PBS and detect the cytokine expression with flow cytometry.

Detection indicators	Fluorophore	Ex (nm)	Em (nm)
CD3	PerCP/Cyanine5.5	440, 480, 675	675
CD4	Elab Fluor® 488	495	520
IFN- $\gamma$	APC	650	660
IL-4	PE	495, 565	575

➤ **For human whole blood sample**

**Note: Sterile technique is required for the step 1-2.**

1. Collect fresh human blood anticoagulated with heparin sodium. Add 250  $\mu$ L of anticoagulated blood and 250  $\mu$ L of RPMI-1640 basal medium (without serum) to each group and mix fully.
2. Refer to the table below to add Cell Stimulation MIX and Protein Transport Inhibitor MIX according to the table below. Incubate at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator for 5 hours, mix every 1-2 hours.

Group	Experimental Content	Addition of Antibodies
Negative group	Untreated	XJH001A
Test group	Add 1 μL Cell Stimulation MIX (500×) and 0.5 μL Protein Transport Inhibitor MIX (1000×)	
Isotype group		XJH001B

**In the subsequent steps, sterile technique is not necessary.**

3. Transfer the cells to 2 mL EP tubes. Add 1 mL of Cell Staining Buffer or  $1 \times$  PBS and mix fully. Centrifuge at  $300 \times g$  for 5 min and discard the supernatant.
4. Resuspend the cells in 100  $\mu$ L of Cell Staining Buffer or  $1 \times$  PBS. Then add 2 mL of  $1 \times$  ACK Lysis Buffer (E-CK-A105) and lyse on ice for 2-3 min. Centrifuge at  $300 \times g$  for 5 min.  
**Note: Lysis is complete when the solution turns from cloudy to clear. Centrifuge promptly to avoid over-lysis and potential cell damage.**
5. Resuspend the cells in 1 mL of Cell Staining Buffer or  $1 \times$  PBS. Centrifuge at  $300 \times g$  for 5 minutes and discard the supernatant.

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- Resuspend the cells in 180  $\mu$ L of Cell Staining Buffer or 1 $\times$  PBS. Add 60  $\mu$ L of Fixation Buffer and mix thoroughly. Fix the cells overnight at 4°C or at room temperature for 1 hour.
- After fixation, the cells will settle at the bottom of the EP tube. Discard the supernatant. Add 1~2 mL of 1 $\times$  Permeabilization Buffer, mix fully, and centrifuge at 500 $\times$ g for 5 min. Discard the supernatant.
- Resuspend the cells in 100  $\mu$ L of 1 $\times$  Permeabilization Buffer. Add 10  $\mu$ L of the antibody to each group according to the table above. Incubate at room temperature with shading light for 60 min, mix every 15~20 min.
- After incubation, add 1 mL of Cell Staining Buffer or 1 $\times$  PBS. Mix fully and centrifuge at 500 $\times$ g for 5 min. Discard the supernatant.
- Resuspend the cells in 100~200  $\mu$ L of Cell Staining Buffer or 1 $\times$  PBS and detect the cytokine expression with flow cytometry.

Detection indicators	Fluorophore	Ex (nm)	Em (nm)
CD3	PerCP/Cyanine5.5	440, 480, 675	675
CD4	Elab Fluor® 488	495	520
IFN- $\gamma$	APC	650	660
IL-4	PE	495, 565	575

## Cautions

- This product is for research use only.
- Permeabilization Buffer (5 $\times$ ) may cause precipitation, which is normal and does not affect the effectiveness.
- When collecting fresh human whole blood samples, it is recommended to use heparin sodium as an anticoagulant. EDTA or similar calcium-chelating anticoagulants may affect cytokine secretion.
- For optimal results, separate PBMCs from fresh human blood within 1 hour of collection. The Ficoll gradient centrifugation should be performed at 20 $\pm$ 2°C. Higher temperatures may reduce cell yield, while lower temperatures may lead to incomplete separation and reduced purity.
- During the PBMC separation process, the blood should be thoroughly mixed before be added to ficoll lymphocyte separation solution. When adding the blood, it is important to avoid disturbing the layered liquid surface to ensure a good interface is formed, otherwise it may affect the separation results.
- High acceleration and deceleration speeds during centrifugation can cause cell loss. It is recommended to set the acceleration to no more than 3 and the deceleration to no more than 2 (i.e., Acc $\leq$ 3, Dec $\leq$ 2).
- Fluorescent substances are prone to quenching. Protect them from light during handling and storage.
- For your safety and health, please wear laboratory overalls and disposable gloves for operation.

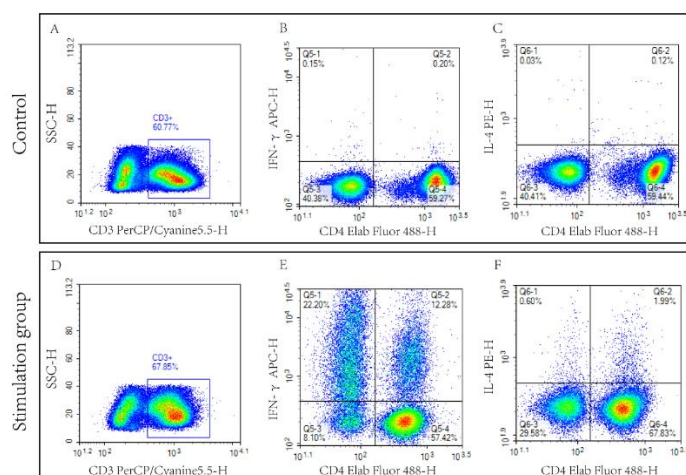
## Common Problems and Solutions

Common Problem	Possible Cause	Suggestion
Contamination of lymphocyte layer with red blood cells	Experimental temperature were too cold.	Allow samples and reagents to reach 20 $\pm$ 2°C before starting the experiment.
	Centrifugal speed was too low.	Increase the centrifugation speed appropriately.

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	Centrifugation time was too short.	Increase the centrifugation time appropriately.
Low lymphocyte viability and yield	Excessive temperature	Allow samples and reagents to reach 20±2°C before starting the experiment.
	The storage time of blood was too long.	Use fresh blood for separation to ensure lymphocyte viability.
Contamination with granulocytes, etc.	Centrifuge was not balanced during centrifugation.	The centrifuge should be leveled before use.
	Sudden stop of the centrifuge causing mixing of lymphocyte layer and lower layers.	Set the centrifuge to decelerate slowly (Dec ≤ 2) to allow gradual stopping.
No cytokine expression detected	Excessive cell density.	Adjust cell density to 1~2×10 <sup>6</sup> cells/mL.
	Interference from red blood cells.	Use human peripheral blood lymphocyte separation medium correctly or use a red blood cell lysis method to eliminate interference.
	Reagents expired.	Store reagents properly and use them within expiration date.
	Poor cell fixation/permeabilization.	Control fixation/permeabilization time according to the instructions.
	Insufficient induction time.	Conduct a preliminary experiment by setting gradient induction time to explore the optimal reaction time.
Excessive intracellular cytokine expression	Poor cell condition, high number of dead cells.	Ensure cells are in good condition prior to induction and exclude dead cells.
	Non-specific antibody binding.	Increase antibody blocking steps to reduce non-specific staining.
Weak fluorescence signal in flow cytometry	Insufficient amount of antibody used.	Add the antibody according to the instructions.
	Insufficient antibody staining time.	Extend antibody incubation time according to the instructions or as needed.
	Too many cells.	Reduce cell density.
	Very low target protein expression level.	Enrich target cells before induction and detection.

## Typical results



**Figure 1:**Flow cytometric analysis of Human Th1/Th2 Flow Cytometry Staining Kit. Normal PBMC cells (Control) and PBMC cells stimulated with Cell Stimulation MIX (Stimulation group). CD3<sup>+</sup>CD4<sup>+</sup>IFN-γ<sup>+</sup> cells (Q5-2) were gated to analyze Th1 and CD3<sup>+</sup>CD4<sup>+</sup>IL-4<sup>+</sup> cells (Q6-2) were gated to analyze Th2.

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