MULTI SCIENCES

联科生物

Human Th1/Th2 Staining Kit

人 Th1/Th2 染色试剂盒

This package insert must be read entirely before using this product. For proper performance, use the insert provided with each individual product received.

Catalog Number

KTH101 - 25

KTH101 - 100

Optimization for human Th1/Th2 staining in anticoagulated blood.

已优化的人抗凝血的Th1/Th2染色。

For research use only. Not for use in diagnostic procedures.

仅用于科研，不得用于临床诊断

INTRODUCTION

The kit is designed to detect frequencies of Th1 and Th2 cells in T lymphocytes (or $CD4^+$ helper T cells) in human anticoagulated blood. Th1, Th2 and Th17 cells are subsets differentiated from helper T cells (strictly speaking, that is Th0 cells) under physiological and pathological conditions. There are extremely few Th1, Th2 or Th17 cells in peripheral blood in a resting state (unstimulated state, e.g. normal physiological state in human) due to the weak differentiation ability of Th0 cells, thus, it's hardly to detect IFN-γ, IL-4 and IL-17A. Th0 cells could differentiate to Th1, Th2 or Th17 cells dependent on different cytokines when stimulated by external factors (e.g. stimuli, pathogens), and more IFN-γ, IL-4 and IL-17A could be detected. In experiment, it is to measure the ability of Th cells in response to stimuli.

Generally, Phorbol 12-Myristate 13-Acetate (PMA) and Ionomycin are used as stimuli. PMA, a PKC activator, could activate phosphorylation of many protein kinases in downstream, cascade reaction induces protein expression, then results in activation of T cells. PKC could be activated in the combination of DAG and Ca^{2+} in cells, with the involvement of Ionomycin (a calcium transporter, transport Ca^{2+} from organelles to cytoplasm), PKC could be further activated in T cells. So PMA and Ionomycin could activate T cells synergistically.

Activated T cells would secrete cytokines to extracellular environment, which could not be detected by flowcytometry, thus cytokines should be blocked in the cell. Cytokines are synthesized in the Golgi and some may traffic through the endoplasmic reticulum to be released in soluble form in the extracellular environment. Blocking secretion by disrupting Golgi to cut off the way of cytokine transport. Generally, Brefeldin A and/or Monensin are used as blocker.

Besides, how to choose Th cells by correct gating is important. The surface marker of Th cells is $CD3^+CD4^+$, however, $CD4^+$ cells will decrease obviously, even disappear after PMA stimulation in human anticoagulant blood for 4 hours due to endocytosis of CD4 molecules induced by PMA. Generally, we use CD3 and CD8 to gate $CD4^+$ cells, that is, $CD3^+CD8^-$ cells would be regarded as $CD3^+CD4^+$ cells.

The choice of anticoagulant would influence the assay. It will not affect activation of Th cells and cytokine secretion when using heparin sodium or heparin lithium as anticoagulant in anticoagulated blood, while using EDTA or sodium citrate as anticoagulant, no cytokines could be detected. In this case, PBMCs should be used for stimulation rather than anticoagulated blood.

LIMITATIONS OF THE PRODUCT

The product is intended for flowcytometry applications. FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

The product should not be used beyond the expiration date on the label.

Do not mix reagents with those from other lots or sources.

产品介绍

本产品用于检测人抗凝血中 Th1 和 Th2 细胞在 T 淋巴细胞 (或 $CD4^+$辅助 T 细胞)中的比例。通常所说的 Th1、Th2 和 Th17 是指在各种生理与病理条件下有能力分化为 Th1、Th2 和 Th17 的 T 辅助细胞 (严格意义上是 Th0 细胞)。静息状态 (即未受任何刺激，如人的正常生理状态)下，Th0 分化为 Th1、Th2 和 Th17 的能力非常弱，所以外周血中仅含有极少量的 Th1、Th2 和 Th17 细胞，这时所能检测到的 IFN-γ、IL-4 和 IL-17A 也微乎其微。而当 Th 细胞受到外界因素 (如刺激素、病原体等)刺激，其中 Th0 即会向 Th1、Th2 或 Th17 分化，具体分化趋向取决于细胞因子的种类。此时，检测到的 IFN-γ、IL-4 或 IL-17A 也较多。实验中检测的 Th1、Th2 和 Th17 实际上是检测 Th 细胞对刺激素刺激的反应能力。

通常选用的刺激素为 Phorbol 12-Myristate 13-Acetate (PMA，佛波酯)和 Ionomycin (离子霉素)。其中 PMA 为 PKC (蛋白激酶 C)的激活物，PKC 则可激活下游众多的蛋白激酶的磷酸化，形成级联反应，导致许多蛋白的表达，进而引起 T 细胞的活化。在细胞内，PKC 可被 DAG (二脂酰甘油)和 Ca^{2+}的共同作用而激活，因此在 Ionomycin (Ca^{2+}的转运剂，可将细胞器内的 Ca^{2+}转运至胞浆内)的参与下，T 细胞内 PKC 可被进一步激活。可见，PMA 与 Ionomycin 协同活化 T 细胞。

活化的 T 细胞可分泌多种细胞因子至细胞外，而流式细胞仪仅能检测细胞内的抗原，所以应将细胞因子阻断在胞内。细胞因子在高尔基体中合成，某些蛋白通过内质网运输以可溶性形式分泌至细胞外。破坏高尔基体即可切断细胞因子的转运途径，阻断其分泌。通常选用的阻断剂为 Brefeldin A (BFA，布雷非德菌素 A)和/或 Monensin (莫能霉素)。

此外，实验如何正确地设门以选定 Th 细胞是很重要的。Th 细胞的表面标志为 $CD3^+CD4^+$，而当用 PMA 刺激人抗凝血 4 小时，会发现 $CD4^+$的细胞明显减少，甚至消失，这是因为 PMA 会诱发细胞表面 CD4 分子被内吞。所以通常的做法是用 CD3 和 CD8 反设 CD4 细胞，即 $CD3^+CD8^-$的细胞被认为是 $CD3^+CD4^+$的细胞。

抗凝剂的选择也会影响检测。使用肝素钠和肝素锂作为抗凝剂，不影响 Th 细胞的活化和细胞因子的分泌，而使用 EDTA 和枸橼酸钠作为抗凝剂则无法检测到细胞因子。此时，应使用 PBMCs 而不是抗凝血进行刺激。

产品的局限

1. 本产品用于流式细胞实验，仅用于科学研究，非诊断试剂，不能用于临床诊断。
2. 请在本产品标记的有效期内使用。
3. 本产品的试剂不能与其他批号的试剂或其他来源的试剂混合使用。

MATERIALS PROVIDED AND STORAGE

Components	Product Code	-25	-100
Anti-Human CD3, FITC (Clone: OKT3)	AH00301	150 µl	600 µl
Anti-Human CD8α, PerCP-Cy5.5 (Clone: RPA-T8)	AH008A07	150 µl	600 µl
Anti-Human IFN-γ, PE (Clone: 4S.B3)	AH0IF04	150 µl	600 µl
Anti-Human IL-4, APC (Clone: 8D4-8)	AH0I405	150 µl	600 µl
PMA/Ionomycin Mixture (250×)	CS1001	30 µl	120 µl
BFA/Monensin Mixture (250×)	CS1002	30 µl	120 µl
FIX & PERM Medium A	GASA	3 ml	12 ml
FIX & PERM Medium B	GASB	3 ml	12 ml
Flow Cytometry Staining Buffer (1×)	S1001	125 ml	125 ml × 3

Note: PMA/Ionomycin Mixture (250×) and BFA/Monensin Mixture (250×) can be stored at - 20℃, FIX & PERM Medium A and Medium B can be stored at room temperature, other reagents can be stored at 2 - 8℃. All reagents can be stable for at least 1 year when stored at recommended condition.

OTHER SUPPLIES REQUIRED

1. **Medium** (e.g. RPMI 1640, DMEM).
2. **Fetal Bovine Serum** (Maybe needed)
3. **Compensation Beads** (Maybe needed)
4. **Lymphocyte separation medium** (Cat No: LSM01, MultiSciences) (Maybe needed)
5. **Paraformaldehyde, 4%** (Cat No: F0001, MultiSciences) (Maybe needed)
6. **12 × 75 mm round bottom test tubes**.
7. **Vortexer.**
8. **CO_2 incubator**.
9. **Swing-out horizon centrifuge** (with rotor for 15 ml tubes).

提供的材料和贮存

组分	编号	-25	-100
抗人 CD3, FITC (克隆号：OKT3)	AH00301	150 µl	600 µl
抗人 CD8α, PerCP-Cy5.5 (克隆号：RPA-T8)	AH008A07	150 µl	600 µl
抗人 IFN-γ, PE (克隆号：4S.B3)	AH0IF04	150 µl	600 µl
抗人 IL-4, APC (克隆号：8D4-8)	AH0I405	150 µl	600 µl
佛波酯/离子霉素混合物 (250×)	CS1001	30 µl	120 µl
布雷非德菌素 A/莫能霉素混合物 (250×)	CS1002	30 µl	120 µl
固定破膜剂试剂 A	GASA	3 ml	12 ml
固定破膜剂试剂 B	GASB	3 ml	12 ml
流式染色缓冲液 (1×)	S1001	125 ml	125 ml × 3

注：佛波酯/离子霉素混合物(250×)和布雷非德菌素A/莫能霉素混合物(250×)可保存于- 20℃，固定破膜剂试剂A和试剂B可保存于室温，其它试剂可保存于2 - 8℃。所有试剂在推荐的条件下可保存至少1年。

未提供的材料设备

1. 培养基 (如 RPMI 1640、DMEM)
2. 胎牛血清 (也许需要)
3. 补偿调节微球 (也许需要)
4. 淋巴细胞分离液 (Cat No: LSM01, MultiSciences) (也许需要)
5. 4% 多聚甲醛 (Cat No: F0001, MultiSciences) (也许需要)
6. 12 × 75 mm 圆底流式管
7. 震荡器
8. CO_2 恒温培养箱
9. 水平离心机 (配 15 ml 离心管的转子)

PROTOCOL

Sample collection

Collect appropriate amount of anticoagulant blood by using heparin as anticoagulant or other anticoagulants (e.g. EDTA, sodium citrate), store at room temperature or 2 - 8℃, and detect within 24 hours.

Sample preparation

1a. *For anticoagulant blood with heparin as anticoagulant*, pipet 125 µl anticoagulant blood into 12 × 75 mm round bottom test tubes, add 125 µl medium without FBS and 1 µl PMA/Ionomycin Mixture (250×) and 1 µl BFA/Monensin Mixture (250×). Mix 125 µl anticoagulant blood and 125 µl medium, served as control. Mix well, incubate in CO_2 incubator at 37℃ for 4 - 6 hours, vortex every 1 - 2 hours during incubation.

1b. *For anticoagulant blood with other anticoagulant (e.g. EDTA, sodium citrate)*, isolate peripheral blood mononuclear cells (PBMCs) by using Lymphocyte separation medium (Cat No: LSM01, MultiSciences). Resuspend pellet at 1 × 10^7/ml in medium with 10 % FBS. Pipet 250 µl PBMCs into 12 × 75 mm round bottom test tubes, add 1 µl PMA/Ionomycin Mixture (250×) and 1 µl BFA/Monensin Mixture (250×). With PBMCs only as control. Mix well, incubate in CO_2 incubator at 37℃ for 4 - 6 hours, vortex every 1 - 2 hours during incubation.

Note: PMA/Ionomycin Mixture (250×) and BFA/Monensin Mixture (250×) are extremely volatile, tighten immediately the lid after use.

2. Pipet 100 µl sample or control into new test tubes, add 5 µl Anti-Human CD3, FITC and 5 µl Anti-Human CD8α, PerCP-Cy5.5 to each tube. Vortex to mix well, and incubate at room temperature for 15 minutes, protect from light.
3. Add 100 µl FIX & PERM Medium A to each tube. Vortex to mix well, and incubate at room temperature for 15 minutes, protect from light.
4. Add 3 ml pre-cool 1× Flow Cytometry Staining Buffer to each tube. Centrifuge at 300 × g for 5 minutes, discard supernant.

   Note: Discard supernant as much as possible, with little residual liquid left.
5. Add 100 µl FIX & PERM Medium B, 5 µl Anti-Human IFN-γ, PE and 5 µl Anti-Human IL-4, APC to each test tube. Vortex to mix well, and incubate at room temperature for 15 minutes, protect from light.

实验步骤

样本收集

使用肝素或其它抗凝剂 (如 EDTA、枸橼酸钠)作为抗凝剂，收集适量抗凝血，室温或 2 - 8℃保存，并于 24 小时内检测。

样本制备

1a. *对于肝素抗凝血*，取 125 µl 抗凝血至流式管中，加入 125 µl 不含血清的培养基和 1 µl PMA/Ionomycin Mixture (250×) 和 1 µl BFA/Monensin Mixture (250×)。取 125 µl 抗凝血和 125 µl 不含血清的培养基，作为对照。混匀，37℃孵育 4 - 6 小时，每隔 1 - 2 小时取出震荡混匀。

1b. *对于使用其它抗凝剂 (如 EDTA、枸橼酸钠)抗凝血*，用淋巴细胞分离液 (Cat No: LSM01, 联科生物) 分离外周血单个核细胞 (PBMCs)。用含 10 %胎牛血清的培养基重悬沉淀，使细胞浓度为 1 × 10^7/ml。取 250 µl PBMCs 至流式管中，加入 1 µl PMA/Ionomycin Mixture (250×)和 1 µl BFA/Monensin Mixture (250×)。以只含 PBMCs 的样本作为对照。混匀，37℃孵育 4 - 6 小时，每隔 1 - 2 小时取出震荡混匀。

注： PMA/Ionomycin Mixture (250×)和 BFA/Monensin Mixture (250×)极易挥发，使用后请及时旋紧管盖。

2. 从样本管和对照管中取 100 µl 细胞悬液至新的流式管中，加入 5 µl Anti-Human CD3, FITC 和 5 µl Anti-Human CD8α, PerCP-Cy5.5。震荡混匀，室温避光孵育 15 分钟。
3. 每管加入 100 µl FIX & PERM Medium A，震荡混匀，室温避光孵育 15 分钟。
4. 每管加入2 ml预冷1× Flow Cytometry Staining Buffer，300 × g离心5分钟，弃上清。

   注： 液体尽量倒干净，不要有残留。
5. 每管加入 100 µl FIX & PERM Medium B、5 µl Anti-Human IFN-γ, PE 和 5 µl Anti-Human IL-4, APC。震荡混匀，室温避光孵育 15 分钟。

6. Add 2 ml 1× Flow Cytometry Staining Buffer to each tube. Centrifuge at 300 × g for 5 minutes, discard supernant.
7. Resuspend pellet by adding 500 µl 1× Flow Cytometry Staining Buffer to each tube, ready for determination. Or add 500 µl 1 - 4 % paraformaldehyde to resuspend pellet, then store at 2 - 8℃, protect from light, determine within 24 hours.

Detection by flowcytometry

1. Voltage and compensation adjustment
   It is recommended to adjust voltage and compensation of flow cytometer by using compensation beads. It is important to adjust voltage of FSC/SSC and gate target cells because compensation beads are often smaller than cells.
2. Correct gating to determine the frequencies of Th1/Th2 cells in $CD3^+CD8^-$ T cells.
   Note: Acquire at least 20,000 to 30,000 $CD3^+CD8^-$ T cells. Depending on the donor, frequencies of cytokine producing cells derived from activation of human PBMCs can vary widely for a particular cytokine. In order to make statistically significant frequency measurements, sufficiently large sample sizes should be acquired during flow cytometric analysis.
   In particular, the number of IL-4 producing cells can be very low or even negligible on PMA/Ionomycin stimulated cells. In these cases, IL-4 polarization cultures should be considered.

Excitation wavelength and emission wavelength of fluorophore

Fluorophore	Ex (nm)	Em (nm)
FITC	495	519
R-Phycoerythrin (PE)	480;565	578
PerCP-Cy5.5	488	682
APC	650	660

6. 每管加入 2 ml 1× Flow Cytometry Staining Buffer，300 × g 离心 5 分钟，弃上清。
7. 每管加入 500 µl 1× Flow Cytometry Staining Buffer 重悬，上机检测；或者加入 500 µl 1 - 4 %多聚甲醛重悬，2 - 8℃避光，于 24 小时内检测。

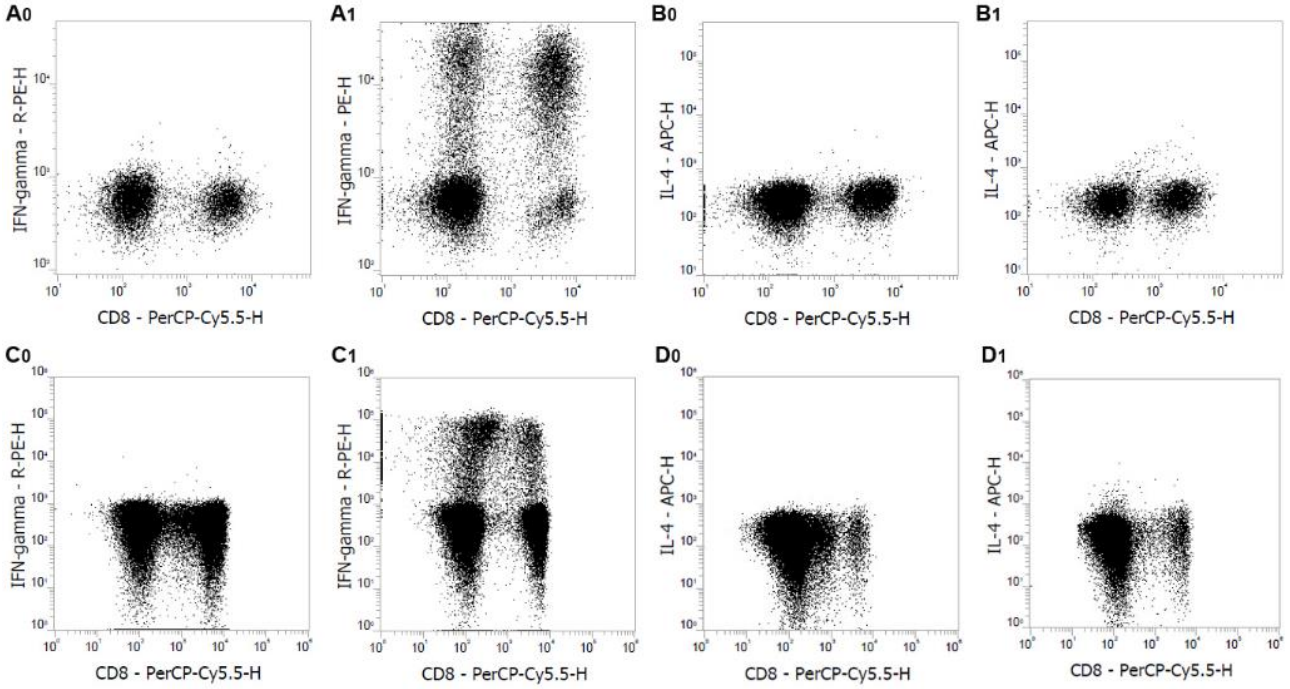
流式检测

1. 调节电压和补偿
   使用补偿微球对流式细胞仪进行电压和补偿调节。补偿微球通常比目的细胞小，在调节 FSC/SSC 电压和细胞设门时需要注意。
2. 正确设门以得到 Th1/Th2 细胞在 $CD3^+$或 $CD4^+$辅助 T 细胞中的比例。
   注：至少获取 20,000 - 30,000 $CD3^+CD8^-$ T 细胞。对于某一细胞因子，因人而异，分泌该细胞因子的细胞比例差异很大。为了进行统计学差异比较，请获取足够多的细胞样本。
   需要注意的是，PMA/Ionomycin 刺激的细胞中，分泌 IL-4 的细胞非常低，甚至可忽略。此时，可考虑 IL-4 的极化培养。

荧光素激发波长和发射波长

荧光素	最大激发波长(nm)	最大发射波长(nm)
FITC	495	519
R-Phycoerythrin (PE)	480;565	578
PerCP-Cy5.5	488	682
APC	650	660

Examples of results/结果示例



Flow cytometric analysis of Human Th1/Th2 Staining Kit. *The staining pattern of IFN-γ and IL-4 on resting heparin anticoagulated blood (A_0, B_0) and PBMCs from EDTA anticoagulated blood (C_0, D_0). PMA/Ionomycin stimulated heparin anticoagulated blood (A_1, B_1) and PBMCs from EDTA anticoagulated blood (C_1, D_1). Dot plot analysis are derived from gated $CD3^+/CD8^-$ cells population. Flow cytometry was performed on a Thermo Fisher Attune NxT. The health status of the volunteers is unclear.*

使用 Human Th1/Th2 Staining Kit 进行流式检测。静息的肝素抗凝血(A_0、B_0)和 EDTA 抗凝血来源的 PBMCs (C_0、D_0)染色 IFN-γ和 IL-4。PMA/Ionomycin 刺激的肝素抗凝血(A_1、B_1)和 EDTA 抗凝血来源的 PBMCs (C_1、D_1)染色 IFN-γ和 IL-4。对 $CD3^+/CD8^-$细胞进行设门分析。实验在 Thermo Fisher 公司的 Attune NxT 流式细胞仪上进行。志愿者的健康状况未详。