Simultaneous Measurement of Human FoxP3 and Ki-67 in Cultured PBMCs

Materials and Reagents

Full Name	Short Name	Catalog Number
BD Falcon™ polystyrene 12 x 75-mm tubes	sample tubes	352058
BD Falcon™ 75-cm² cell culture flask	culture flasks	353136
BD Falcon™ conical tubes with screw caps	conical tubes	358206
DMEM, containing 10% FCS	medium	
Sterile 1X PBS	1X PBS	
Ficoll-Paque™ PLUS (GE Healthcare)	Ficoll	17-1440-02
BD Pharmingen™ Human FoxP3 Buffer Set	FoxP3 Buffer Set	560098
BD Pharmingen™ Stain Buffer (BSA)	Stain Buffer	554657
NA/LE anti-Human CD3, clone UCHT1	Anti-CD3	555329
NA/LE anti-Human CD28, clone CD28.2	Anti-CD28	555725
Alexa Fluor® 488 Mouse Anti-Human CD4, clone RPA-T4	CD4-Alexa Fluor® 488	557695
PerCP-Cy™5.5 Mouse Anti-Human CD25, clone M-A251	CD25-PerCP-Cy5.5	560503
Alexa Fluor® 647 Mouse Anti-Human FoxP3, clone 236a/E7	FoxP3-Alexa Fluor® 647	561184
PE Mouse Anti-Human Ki-67 Set	Ki-67-PE	556027

Procedural Notes

- This assay enables the simultaneous measurement of cellular proliferation (using the Ki-67 marker) and cell surface markers in FoxP3-positive cells.
- In this example protocol, the cells were prepared from human whole blood. PBMCs were isolated from human whole blood by Ficoll treatment using a standard protocol. The PBMCs were added to a culture flask at 1 x 10^6 cells/mL of medium.
- Do not store prepared buffers. Diluted buffer A and buffer C must be prepared fresh on the day of the experiment.

Procedure: Stimulation

1. Coat a sterile culture flask with anti-CD3 at a concentration of 10 µg/mL in sterile 1X PBS. Incubate overnight at room temperature (RT).
2. The next day, remove the anti-CD3 and wash once with 2 mL of medium.
3. Prepare PBMCs from human whole blood using a standard protocol for Ficoll isolation.
4. Resuspend the cells and plate at 1 x 10^6 cells/mL of medium onto a culture flask.
5. Add NA/LE anti-CD28 at a concentration of 2 µg/mL and incubate for 72 hours at 37°C in 5% CO_2.
6. In parallel, prepare a mock treated flask using the same protocol but excluding the stimulation antibodies.
7. Harvest the cells after 72 hours. Wash the cells two times with Stain Buffer.
8. Pellet the cells by centrifugation at 250*g* for 10 minutes. Remove the supernatant.
9. Mix the pellet to loosen the cells. Resuspend the cells at 12 x 10^6 cells/mL of Stain Buffer. This yields about 1.2 x 10^6 cells per test, when using 100 µL of cells. Proceed to the staining protocol.

Simultaneous Measurement of Human FoxP3 and Ki-67 in Cultured PBMCs

Procedure: Buffer Preparation (from FoxP3 Buffer Set)

1. Bring all the buffers to room temperature (20°C to 25°C) before use.
2. Prepare a solution of 1X FoxP3 Buffer A by diluting FoxP3 Buffer A (10X concentrate) 1:10 with room temperature, deionized water.
3. Make a working solution of Buffer C by diluting FoxP3 Buffer B into 1X FoxP3 Buffer A at a ratio of 1:50 (Buffer B:Buffer A)

Procedure: Cell Preparation and Staining

1. Aliquot the appropriate amount of selected surface staining reagents into sample tubes.
2. Add 100 µL of cells to each sample tube and mix well. Incubate for 20 minutes at RT in the dark.
3. Wash the cells with 2 mL of Stain Buffer. Pellet the cells by centrifugation at 500*g* for 10 minutes. Remove the supernatant. Mix the pellet to loosen the cells.
4. Fix the cells with 2 mL of freshly prepared cold 1X FoxP3 Buffer A. Mix well. Incubate for 10 minutes at RT in the dark.
5. Pellet the cells by centrifugation at 500*g* for 5 minutes. Remove the fixation buffer. Caution: the pellet is buoyant.
6. Wash the cells with 2 mL of Stain Buffer. Pellet the cells by centrifugation at 500*g* for 5 minutes. Remove the supernatant. Mix the pellet to loosen the cells.
7. Permeabilize the cells by adding 0.5 mL of freshly prepared pre-warmed (room temperature) 1X FoxP3 Buffer C. Vortex to mix. Incubate for 30 minutes at RT in the dark.
8. Wash the cells by adding 2 mL of Stain Buffer. Pellet the cells by centrifugation at 500*g* for 10 minutes at RT. Remove the supernatant, leaving about 100 µL in the tube. Mix the pellet to loosen the cells.
9. Repeat wash step 8.
10. Add 5 µL of FoxP3- Alexa Fluor® 647 antibody and 20 µL of Ki-67-PE to each sample tube, and mix well. Incubate for 20 minutes at RT in the dark.
11. Repeat wash step 8.
12. Resuspend the cells in 0.5 mL of Stain Buffer and analyze immediately.

Flow Cytometric Analysis of Stained Cell Samples

Flow cytometric analysis of the samples can be performed by using a flow cytometer equipped with two lasers: a 488-nm blue laser and a 640-nm red laser.

Simultaneous Measurement of Human FoxP3 and Ki-67 in Cultured PBMCs

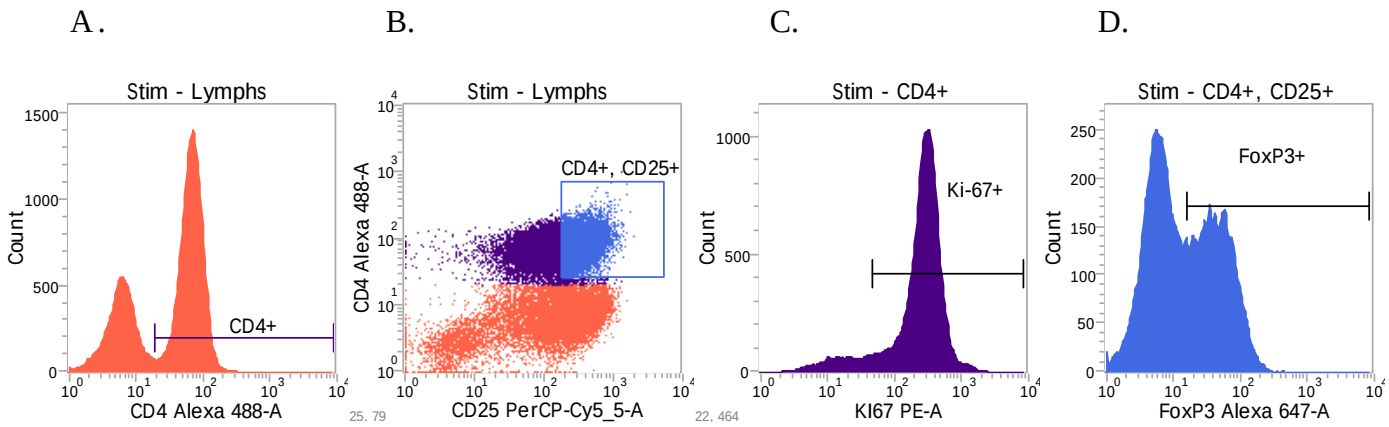

Figure 1. Results from the analysis of stimulated cells. The cells were stimulated with anti-CD3 and anti-CD28 antibodies for 72 hours. NA/LE anti-Human CD3 was coated onto sterile tissue culture plates at a concentration of 10 µg/mL in sterile 1X PBS. Soluble NA/LE anti-Human CD28 was used at 2 µg/mL. Panel C shows that 90% of the $CD4^+$ cells were Ki-67 positive. Panel D shows that 45% of the $CD4^+$, $CD25^+$ cells were $FoxP3^+$.

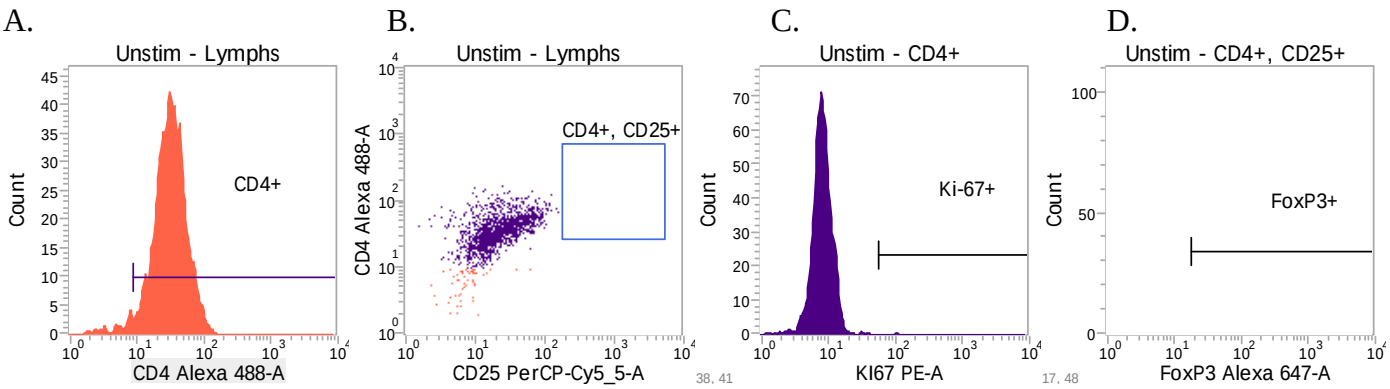

Figure 2. Results from the analysis of unstimulated cells (negative control). Panel C shows an insignificant number of the unstimulated $CD4^+$ cells were Ki-67 positive. Panel D shows an insignificant number of the unstimulated $CD4^+$, $CD25^+$ cells were FoxP3 positive.

The sample acquisition in this example was done by using the BD FACSVerse™ flow cytometer, and the analysis by using BD FACSuite™ software.