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

Materials and Reagents

<table>
<thead>
<tr>
<th>Full Name</th>
<th>Short Name</th>
<th>Catalog Number</th>
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<tbody>
<tr>
<td>BD Falcon™ polystyrene 12 x 75-mm tubes</td>
<td>sample tubes</td>
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<td>BD Falcon™ 75-cm² cell culture flask</td>
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<td>BD Falcon™ conical tubes with screw caps</td>
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<td>DMEM, containing 10% FCS</td>
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<tr>
<td>Sterile 1X PBS</td>
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<td>Ficoll-Paque™ PLUS (GE Healthcare)</td>
<td>Ficoll</td>
<td>17-1440-02</td>
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<td>BD Pharmingen™ Human FoxP3 Buffer Set</td>
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<td>BD Pharmingen™ Stain Buffer (BSA)</td>
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<td>NA/LE anti-Human CD3, clone UCHT1</td>
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<td>NA/LE anti-Human CD28, clone CD28.2</td>
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<td>Alexa Fluor® 488 Mouse Anti-Human CD4, clone RPA-T4</td>
<td>CD4-Alexa Fluor® 488</td>
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<td>CD25-PerCP-Cy5.5</td>
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<td>Alexa Fluor® 647 Mouse Anti-Human FoxP3, clone 236a/E7</td>
<td>FoxP3-Alexa Fluor® 647</td>
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<td>PE Mouse Anti-Human Ki-67 Set</td>
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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 10⁶ 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 10⁶ 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% CO₂.
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 250g for 10 minutes. Remove the supernatant.
9. Mix the pellet to loosen the cells. Resuspend the cells at 12 x 10⁶ cells/mL of Stain Buffer. This yields about 1.2 x 10⁶ cells per test, when using 100 μL of cells. Proceed to the staining protocol.
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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 500g 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 500g 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 500g 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 500g 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.
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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.

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