Simultaneous Measurement of Mouse Foxp3 and Ki-67 in Cultured Splenocytes

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 flask	353136
BD Falcon™ conical tubes with screw cap	conical tube	358206
RPMI-1640 containing 10% FCS	medium	
Sterile 1X PBS	1X PBS	
BD Pharmingen™ Mouse Foxp3 buffer set	Foxp3 Buffer Set	560409
BD Pharm Lyse™ solution	Lysing Solution	555899
BD Pharmingen™ Stain Buffer (BSA)	Stain Buffer	554657
BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution	Fix/Perm Solution	554722
BD Cytofix/Cytoperm™ Perm/Wash™ Buffer	Perm/Wash Buffer	554723
NA/LE anti-mouse CD3e, clone 145-2C11	Anti-CD3	553057
NA/LE anti-mouse CD28, clone 37.51	Anti-CD28	553294
Alex Fluor® 488 Rat Anti-mouse CD4, clone RM4-5	CD4- Alexa Fluor® 488	557667
PerCP-Cy™5.5 Rat Anti-Mouse CD25, clone PC61	CD25-PerCP-Cy5.5	551071
Alexa Fluor® 647 Rat anti-Mouse FoxPp3, clone MF23	Foxp3- Alexa Fluor® 647	560401
PE Mouse Anti-Human Ki-67 Set	Ki-67-PE	556027
Anti-Rat Ig, κ/Negative Control Compensation Particles Set	Compensation beads	552844

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 protocol, the cells were prepared from mouse spleen Balb/c mice (female). The mice were sacrificed, spleen was harvested, and the red blood cells were lysed with Lysing Solution. The splenocytes were added to a culture flask at 1 x 10^6 cells/mL of medium.
- Do not store diluted Fixation Buffer and Permeabilization Buffer prepared from the Foxp3 Buffer Set. Prepare them fresh every time.
- Determine the appropriate amount of the Rat Anti-mouse Foxp3 antibody by titration.

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, discard the anti-CD3 and wash once with 2 mL of medium.
3. Prepare splenocytes from mouse spleen. Lyse the red blood cells for 2 minutes at RT, using 2 mL of Lysing Solution for each spleen.
4. Add 18 mL of RPMI and centrifuge at 250*g* for 5 to 10 minutes. Remove the supernatant.
5. Resuspend the cells and plate at 1 x 10^6 cells/mL of medium onto a culture flask.
6. Add anti-CD28 at a concentration of 2 µg/mL and incubate for 72 hours at 37°C in 5% CO_2.
7. In parallel, prepare a mock treated flask using the same protocol but excluding the stimulation antibodies.
8. Harvest the cells after 72 hours. Wash the cells two times with Stain Buffer.
9. Pellet the cells by centrifugation at 250*g* for 10 minutes. Remove the supernatant.
10. Mix the pellet to loosen the cells. Resuspend the cells at 10 x 10^6 cells/mL of Stain Buffer.
11. Proceed to the Cell Preparation and Foxp3 Staining procedure.

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Procedure: Buffer Preparation (from Foxp3 Buffer Set)

- Prepare 1X Fixation Buffer.
  1. Dilute 1 part Fixation Concentrate with 19 parts 1X PBS.
  2. Prepare 2 mL for each test (1 x 10^6 cells).
  3. Place on ice.
- Prepare 1X Permeabilization Buffer.
  1. Dilute 1 part Permeabilization Concentrate with 4 parts 1X PBS.
  2. Prepare 4 mL for each test (1 x 10^6 cells).
  3. Pre-warm to 37°C before use.

Procedure: Cell Preparation and Foxp3 Staining

1. Aliquot the appropriate amount of selected surface staining reagents to 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 250*g* for 10 minutes.
4. Remove the supernatant. Mix the pellet to loosen the cells.
5. Fix the cells with 2 mL of freshly prepared cold 1X Fixation Buffer. Mix well. Incubate for 30 minutes at 4°C in the dark.
6. Pellet the cells by centrifugation at 500*g* for 5 minutes. Remove the Fixation Buffer.
7. Wash the cells with 2 mL of prepared pre-warmed 1X Permeabilization Buffer.
8. Pellet the cells by centrifugation at 500*g* for 5 minutes.
9. Remove the supernatant. Mix the pellet to loosen the cells.
10. Permeabilize the cells by adding 2 mL of freshly prepared pre-warmed 1X Permeabilization Buffer. Incubate for 30 minutes at 37°C in the dark.
11. Pellet the cells by centrifugation at 500*g* for 5 minutes. Remove the Permeabilization Buffer.
12. Wash the cells with 2 mL of Stain Buffer. Pellet the cells by centrifugation at 500*g* for 10 minutes. Remove the supernatant, leaving about 100 µL in the tube. Mix the pellet to loosen the cells.
13. Add 20 µL of Rat Anti-Mouse Foxp3 antibody (dilute the appropriate amount in Stain Buffer) to each sample tube and mix well. Incubate for 20 minutes at RT in the dark.
14. Wash the cells twice 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.

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Procedure: Ki-67 Staining

1. Add 250 µL of Fix/Perm Solution to each sample tube and mix well. Incubate for 20 minutes at 4°C.
2. Wash the cells twice with 2 mL of 1X Perm/Wash Buffer. Pellet the cells by centrifugation at 500*g* for 10 minutes.
3. Remove the supernatant, leaving about 100 µL in the tube. Mix the pellet to loosen the cells. Note: Perm/Wash Buffer must be maintained in washing steps to keep cells permeabilized.
4. Add 20 µL of Anti-Ki-67 antibody to the sample tubes and mix well. Incubate for 30 minutes in the dark at RT.
5. Wash the cells twice with 2 mL of 1X Perm/Wash Buffer. Pellet the cells by centrifugation at 500*g* for 10 minutes.
6. Remove the supernatant, leaving about 100 µL in the tube. Mix the pellet to loosen the cells.
7. 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 635-nm red laser.

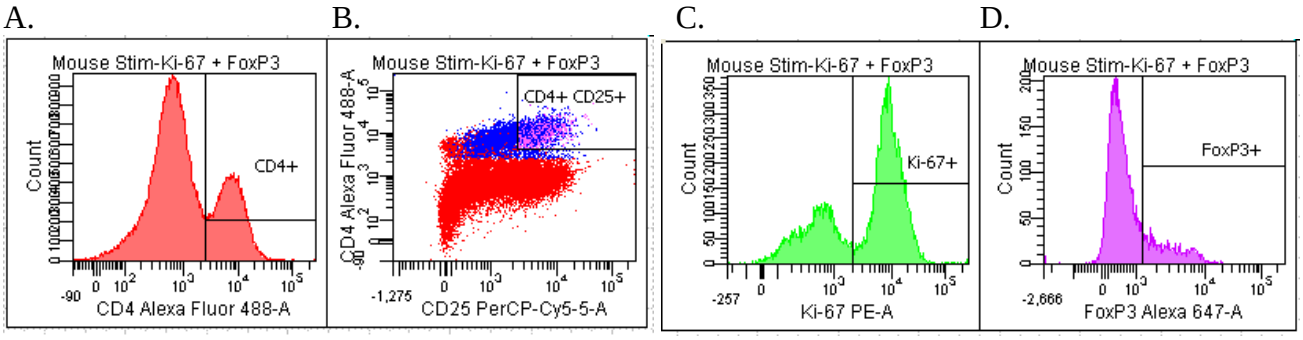

Figure 1. Results from the analysis of stimulated cells. The cells were stimulated with anti-CD3 and anti-CD28 antibodies for 72 hours. NA/LE anti-mouse CD3e was coated onto sterile tissue culture plates at a concentration of 10 µg/mL in sterile 1X PBS. Soluble NA/LE anti-CD28 was used at 2 µg/mL. Panel C shows that 66% of the $CD4^+$ cells were Ki-67^+. Panel D shows that 19% of the $CD4^+$, $CD25^+$ cells were $Foxp3^+$.

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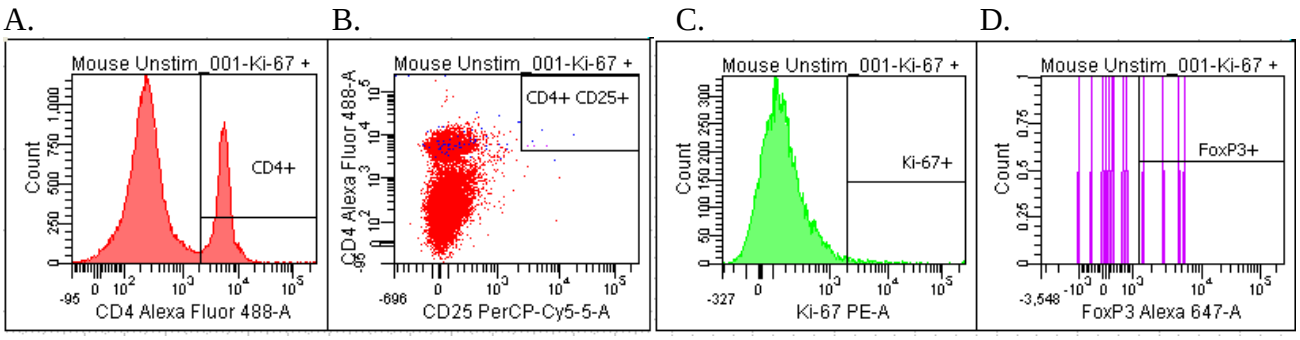

Figure 2. Results from the analysis of unstimulated cells (negative control). Panel C shows an insignificant number of the unstimulated $CD4^+$ cells were Ki-67^+. Panel D shows an insignificant number of the unstimulated $CD4^+$, $CD25^+$ cells were $Foxp3^+$.

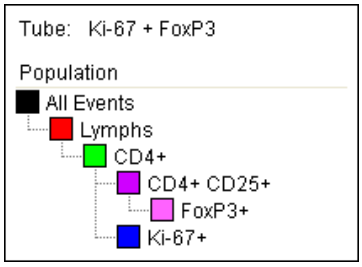

Figure 3. Population hierarchy.

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