

Phenotyping of Human PBMC using Lyoplate

1. Principle

In this protocol, we use lyophilized cocktails of fluorescent-labeled antibodies preconfigured in 96-well plates (Lyoplates) to stain PBMC that have been previously cryopreserved. We use an LSRII flow cytometer to acquire the fluorescence data. Subsequent analysis of the fluorescence data using FlowJo software allows for cell types to be analysed based on cell size, internal complexity, and relative fluorescence intensity in each color. The percentage of each cell type is determined and reported as a percent of the parent cell type.

2. Materials and Equipment

- 2.1. PBMC, fresh or thawed frozen
- 2.2. Complete RPMI (RPMI with 10% FBS, P/S, glutamine)
- 2.3. Benzonase (25X10⁵ U/ml)
- 2.4. 2% Para-formaldehyde (PFA)
- 2.5. FACS buffer (PBS with 2% FBS and 0.1% Na Azide)
- 2.6. Phenotyping antibodies
- 2.7. 37°C water bath
- 2.8. 96-well plate
- 2.9. Biosafety cabinet
- 2.10. Centrifuge
- 2.11. Calibrated pipettes
- 2.12. ViCell or Hemocytometer cell counter

3. Procedure

Thaw PBMC

- 3.1. Warm media to 37°C in water bath. Each sample will require 20ml of media with benzonase. Calculate the amount needed to thaw all samples, and prepare a separate aliquot of warm media with 1:10000 benzonase (25 U/ml). Thaw no more than 10 samples at a time.

- 3.2. Remove samples from liquid nitrogen and transport to lab on dry ice.
- 3.3. Place 10ml of warmed benzonase media into a 15ml tube, making a separate tube for each sample.
- 3.4. Thaw frozen vials in 37°C water bath.
- 3.5. When cells are nearly completely thawed, carry to hood.
- 3.6. Add 1ml of warm benzonase media from appropriately labeled centrifuge tube slowly to the cells, then transfer the cells to the centrifuge tube. Rinse vial with more media from centrifuge tube to retrieve all cells.
- 3.7. Continue with the rest of the samples as quickly as possible.
- 3.8. Centrifuge cells at 1400 rpm (RCF=390) for 8 minutes at room temperature.
- 3.9. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
- 3.10. Gently resuspend the pellet in 1ml warmed benzonase media. Filter cells through a 70 micron cell strainer if needed (i.e. if you observe any clumps). Add 9ml warmed benzonase media to the tube to make volume 10ml altogether.
- 3.11. Centrifuge cells at 1400 rpm (RCF=390) for 8 minutes at room temperature.
- 3.12. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
- 3.13. Resuspend cells in 0.5ml FACS buffer.
- 3.14. Count cells with Vicell (or hemocytometer). To count, take 20ul cells and dilute with 480ul PBS in vicell counting chamber. Load onto vicell as PBMC with a 1:25 dilution factor.
- 3.15. Adjust the cell concentration to 10×10^6 cells/ml with FACS buffer. Note-it is typical to recover $4-8 \times 10^6$ cells from a vial frozen at 10×10^6 cells/vial. Up to 10×10^6 cells, no need to dilute more, just leave in 0.5 ml FACS buffer.

Stain cells

- 3.16 Add 50-60 μ l of cells from each donor into the first 7 wells of each sample row (C-H) on the Lyoplate. [Note that you can cut the foil seal so as to only expose the rows you are using, if phenotyping fewer than 6 donors at once]. Add cells to the side of each well

- without touching pellets and let sit for 2-3 mins, then mix up and down 5-6 times using a multichannel pipette. Avoid creating bubbles.
- 3.17 Combine 10 drops each of positive and negative BD anti-mouse Ig CompBeads (blue and white cap dropper vials) in an empty FACS tube. Add 50 μ l of the combined beads to all the compensation wells (row A and well B1).
 - 3.18 Add 10 μ L each of CD85j FITC and CD28 PE antibodies into each sample well of column 1 to complete cocktail 1. Add 10 μ L each of TCR $\gamma\delta$ FITC and PD-1 PE to each sample well of column 7 to complete cocktail 7.
 - 3.19 Incubate 45-60 minutes at room temperature with gentle shaking.
 - 3.20 Add 150 μ l of FACS buffer, without pipetting up and down, and spin at 1200 rpm, 2min, @ 4°C, for the first wash. Aspirate, resuspend in 200 μ l FACS buffer & spin 1200 rpm, 2 min, @ 4°C, for a second wash. Repeat washing with 200 μ l FACS buffer for a third and fourth wash. After the 4th wash, resuspend wells in 200 μ L FACS buffer, cover with foil and keep at 4°C until ready to run on LSR II.
 - 3.21 Acquire data on LSR II. Create a new blank experiment using baseline CST settings, apply Lyoplate application settings, and apply the appropriate plate template. Specific sample ID's are added into the plate template prior to acquiring data. Record the compensation specimen, then calculate compensation, and record the experimental samples (Record 20,000-50,000 viable events/lymphocytes per well for each experimental sample).

Analyze Data

- 3.22 Analyze data using FlowJo software using appropriate templates created for each of the seven tubes. Adjust gates on a donor-specific basis, if necessary, to control for any differences in background or positive staining intensity.
- 3.23 Export the statistics for each gated population to an Excel spreadsheet.

Stanford Human Immune Monitoring Core
Stanford, CA 94305
Phone: 650-723-5050

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	CD161 FITC	CD24 PE	HLA-DR PerCP-Cy5.5	CD45RA PE-Cy7	CD16 PE-Cy7	CD38 PE-Cy7	CD33 PE-Cy7	CD3 V450	CD27 APC	CD127 Alexa 647	CD8 APC-H7
B	CD20 APC-H7											
C	Cocktail 1	Cocktail 2	Cocktail 3	Cocktail 4	Cocktail 5	Cocktail 6	Cocktail 7					
D	Cocktail 1	Cocktail 2	Cocktail 3	Cocktail 4	Cocktail 5	Cocktail 6	Cocktail 7					
E	Cocktail 1	Cocktail 2	Cocktail 3	Cocktail 4	Cocktail 5	Cocktail 6	Cocktail 7					
F	Cocktail 1	Cocktail 2	Cocktail 3	Cocktail 4	Cocktail 5	Cocktail 6	Cocktail 7					
G	Cocktail 1	Cocktail 2	Cocktail 3	Cocktail 4	Cocktail 5	Cocktail 6	Cocktail 7					
H	Cocktail 1	Cocktail 2	Cocktail 3	Cocktail 4	Cocktail 5	Cocktail 6	Cocktail 7					

COCKTAIL 1 T cells

HOLD
HOLD
CD4 PERCP-CY5.5
CD45RA PE-CY7
CD27 APC
CD8 APC-H7
CD3 V450

COCKTAIL 2 NK/NKT

CD94 FITC
CD314 PE
HLA-DR PERCP-CY5.5
CD16 PE-CY7
CD56 APC
CD8 APC-H7
CD3 V450

COCKTAIL 3 B cells

Anti-IgD FITC
CD24 PE
CD19 PERCP-CY5.5
CD38 PE-CY7
CD27 APC
CD20 APC-H7
CD3 V450

COCKTAIL 4 Treg

CD161 FITC
CD25 PE
CD4 PERCP-CY5.5
CD45RA PE-Cy7
CD127 ALEXA 647
CD8 APC-H7
CD3 V450

COCKTAIL 5 FMO CXCR3

CD56 FITC
CD16 FITC
HOLD
CD4 PERCP-CY5.5
CD33 PE-CY7
CD19 APC
CD8 APC-H7
CD3 V450

COCKTAIL 6 CXCR3+subsets

CD56 FITC
CD16 FITC
CXCR3 PE
CD4 PERCP-CY5.5
CD33 PE-CY7
CD19 APC
CD8 APC-H7
CD3 V450

COCKTAIL 7 Activated T

HOLD
HOLD
CD4 PERCP-CY5.5
CD38 PE-CY7
HLA-DR APC
CD8 APC-H7
CD3 V450