

Intracellular cytokine staining on PBMCs

1. Principle

Production of cytokines plays an important role in the immune response. Cytokines are involved in many different pathways including the induction of many anti-viral proteins by IFN gamma, the induction of T cell proliferation by IL-2 and the inhibition of viral gene expression and replication by TNF alpha. Cytokines are not preformed factors but are rapidly produced and secreted in response to cellular activation.

Intracellular cytokine detection by flow cytometry has emerged as the premier technique for studying cytokine production at the single-cell level. It detects the production and accumulation of cytokines within the endoplasmic reticulum after cell stimulation, allowing direct TH1 versus TH2 determination. It can also be used in combination with other flow cytometry protocols for immunophenotyping using cell surface markers or with MHC multimers to detect an antigen specific response, making it an extremely flexible and versatile method. This capability, combined with the high throughput inherent in the instrumentation, gives intracellular cytokine staining an enormous advantage over existing single-cell techniques such as ELISPOT, limiting dilution, and T cell cloning. The principle steps of intracellular cytokine staining is as follows:

- Cells are activated for a few hours using either a specific peptide or a non-specific activation cocktail

- An inhibitor of protein transport (e.g. Brefeldin A) is added to to retain the cytokines within the cell
- Next, EDTA is added to remove adherent cells from the activation vessel
- After washing, antibodies to cell surface markers can be added to the cells
- The cells are then fixed in paraformaldehyde and permeabilized
- The anti-cytokine antibody is added and the cells can be analyzed by flow cytometer.

2. Materials and Equipment

- 2.1. PBMC, fresh or thawed frozen
- 2.2. Complete RPMI (RPMI, 10% FBS, Pen-Strep, glutamine)
- 2.3. Benzonase (Sigma Catalog # B7651)
- 2.4. PMA (Sigma Catalog # P8139)
- 2.5. Ionomycin (Calbiochem Catalog # 407952)
- 2.6. Dynabeads Human T Activator CD3/CD28 (Invitrogen Cat # 111.32D)
- 2.7. Brefeldin A (Sigma Catalog # B7651)
- 2.8. Monensin, 1000x (Biolegend Catalog # 420701)
- 2.9. 0.5M EDTA (Sigma Catalog # E-5134)
- 2.10. LIVE/DEAD® Fixable Red Dead Cell Stain (Invitrogen Cat # L23102)
- 2.11. FACS buffer (PBS with 2% FBS and 0.1% Na Azide)
- 2.12. BD FACS Lysing Solution, 10X (Catalog # 349202)
- 2.13. BD FACS Permeabilizing Solution 2, 10x (Catalog # 347692)

- 2.14. fluorochrome-linked surface markers (e.g. CD3, CD4, CD8)
- 2.15. fluorochrome-linked cytokine antibodies (e.g. IFN-gamma, IL-2, TNF)
- 2.16. 96- deep well V-bottom plates
- 2.17. Falcon round-bottom FACS tubes
- 2.18. Magnet for Dynabead separation (BD I Mag Catalog # 552311)
- 2.19. 37°C water bath
- 2.20. Biosafety cabinet
- 2.21. Centrifuge
- 2.22. CO₂ incubator at 37°C
- 2.23. Calibrated pipettes

3. Procedure

Thaw PBMC

- 3.1. Warm media to 37°C in water bath. Each sample will require 22ml 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 3 samples at a time. Run one control PBMC with each batch of samples.
- 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 1500 rpm 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. Add 9 ml more warmed benzonase media to the tube.
- 3.11. Centrifuge cells at 1500 rpm for 8 minutes at room temperature. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
- 3.12. Resuspend cells in 1ml warm media.
- 3.13. Count cells with Vicell (or hemocytometer if necessary). To count, take 20 ul cells and dilute with 480 ul PBS in vicell counting chamber. Load onto Vicell as PBMC with a 1:25 dilution factor.
- 3.14. Adjust the cell concentration to $5-10 * 10^6$ cells/ml with warm media (no more benzonase at this point.)
- 3.15. Using a multichannel pipette, add 200 μ l cells ($1 * 10^6$ cells) into each well of a 96-well deep well plate. Split each sample into two or more wells keeping one as an unstimulated control and the others for different types of stimulation.
- 3.16. Rest overnight (6-18h) at 37°C in CO₂ incubator.

Stimulate Cells

3.17. After overnight rest at 37°C, add the activation reagents and secretion inhibitor (Brefeldin A/ Monensin) to the well for stimulation. Add only the secretion inhibitor to the unstimulated control well.

Reagent	Stock Concentration	Intermediate Dilution	Final Concentration
phorbol12-myristate13acetate (PMA)	1 mg/mL in (store in aliquots at -20C)	1:1000 in PBS	10 ng/ml
Phytohemagglutinin (PHA)	1 mg/mL in (store at 4C)	1:10 in PBS	1 ug/ ml
SEB	50 µg/mL in PBS	None	1 µg/mL (1:50)
Peptide mixes	0.5-1 mg/mL/pep in DMSO (store in aliquots at -20C)	1:10 in PBS	1 µg/mL/peptide (1:50 - 1:100)
Brefeldin A	5 mg/mL in DMSO (store in aliquots at -20C)	1:10 in PBS	10 µg/mL (1:50) or 5µg/mL (1:100) with monensin
Monensin	5 mg/mL in ethanol (store at -20C)	1:10 in PBS	10 µg/mL (1:50) or 5 µg/mL (1:100) with brefeldinA
Anti-CD3/ CD28	Follow manufacturer instruc	-	-

Note: ¹ It is important to avoid solvent toxicity. Final DMSO+ethanol concentration from all sources (peptides, brefeldin A, monensin) should not exceed 0.5%.

² For most cytokines: use brefeldin A at 10 µg/mL final concentration (see stock preparation table). For CD107 and CD154: use monensin at 10 µg/mL final concentration (see stock preparation table). For assays combining cytokines and CD107 or CD154: use brefeldin A and monensin at 5 µg/mL final concentration each.

³ Addition of costimulatory antibodies is optional. Add 1 µg/mL final concentration of CD28 and/or CD49d (labeled antibody can be used if analysis of the marker is desired).

3.18. Incubate the cells for 4 hours (PMA+Ionomycin stimulation, PHA+Ionomycin stimulation) or 6-8 hours (anti-CD3/CD28 stimulation, peptide stimulation) at 37°C, in a CO₂ incubator.

Note: For most cytokines 6-12 h incubation at 37°C is sufficient; For IL-10 and TGF incubate for 12-24 h

- 3.19. Add EDTA to a final concentration of 2 mM and incubate for 15 min at room temperature.
- 3.20. Wash the cells with PBS at 1600 rpm, 8 min at room temperature.

Staining

- 3.21. Repeat PBS wash at 1600 rpm, 8 min, room temperature and resuspend the cells in 500 ul PBS.
- 3.22. Stain with Red LIVE/DEAD cell viability dye (L12302, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.
- 3.23. Incubate at room temperature for 30 min in dark.
- 3.24. Centrifuge the cells at 1600 rpm, 8 min, room temperature.
- 3.25. Discard supernatant and wash cells with PBS at 1600 rpm, 8 min, room temperature.
- 3.26. Repeat another wash in FACS buffer (PBS supplemented with 2% FBS), at 1600 rpm, 8 min, room temperature.
- 3.27. Decant supernatant and resuspend cells in residual volume.
- 3.28. Prepare the surface staining cocktail according to titre per test for each antibody (pre-determined/ provided by manufacture). See an example below for staining ten tubes/wells.

Antibody panel	Stain	Titre(ul/ sample)	X # of samples	Total ul
CD3	V500	5	10	50
CD4	PerCPCy5.5	10	10	200
CD8	V450	5	10	50

- 3.29. Add calculated volume of staining cocktail for each sample to (20 ul in the example above). Add appropriate amount of single antibodies to beads for compensation controls.
- 3.30. Incubate cells for 30 min at room temperature in the dark.
- 3.31. Add 2ml FACS buffer to each well / tube.
- 3.32. Centrifuge the cells at 1600 rpm, 8 min, room temperature.
- 3.33. Discard supernatant and wash cells again with FACS buffer at 1600 rpm, 8 min, room temperature.
- 3.34. Prepare 1x BD FACS Lysing Solution (10x diluted to 1x in water).
- 3.35. Add 2 ml per tube / well, mix well and incubate the cells for 10 min at room temperature, in the dark.
- 3.36. Centrifuge cells at 2000 rpm for 8 min at 4°C and discard supernatant.
- 3.37. Wash cells 2X in FACS buffer.
- 3.38. Prepare cold 1x BD FACS Perm-2 buffer (10x diluted to 1x in water).
- 3.39. Resuspend the cell pellet in 500 uL of 1x FACS Permeabilizing Solution (prepared above) and incubate for 30 min on ice in dark.
- 3.40. Centrifuge cells at 2000 rpm for 8 min at 4°C and discard supernatant.
- 3.41. Wash cells 2X in FACS buffer at 4°C.
- 3.42. Decant supernatant and resuspend cells in residual volume.
- 3.43. Prepare the intracellular staining cocktail according to titre per test for each antibody (pre-determined / provided by manufacture). See example below for staining ten tubes/wells.

Antibody panel	Stain	Titre(ul/ sample)	X # of samples	Total ul
IFN- γ	FITC	20	10	200
IL-17	PE	20	10	200
IL-4	APC	5	10	50
TNF α	Alexa700	1.2	10	12
IL-2	PE-Cy7	5	10	50

- 3.44. Calculate and add required volume of staining cocktail to each sample (51.2 ul in the example above) and incubate cells for 30 min – 60 min, on ice, in the dark.
- 3.45. Add 2ml FACS buffer to each well / tube.
- 3.46. Centrifuge cells at 2000 rpm for 8 min at 4°C and discard supernatant.
- 3.47. Wash cells 2X in FACS buffer at 4°C.
- 3.48. Discard supernatant and resuspend in a final volume of 150 ul FACS buffer for Flow.