Cytokine-Stimulated Phosphoflow on PBMC

1. Principle

Phosphorylation of tyrosine, serine, and threonine residues is critical for the control of protein activity involved in various cellular events. An assortment of kinases and phosphatases regulate intracellular protein phosphorylation in many different cell signaling pathways, such as T and B cell signaling, those regulating apoptosis, growth and cell cycle control, plus those involved with cytokine, chemokine, and stress responses. Phosphoflow assays combine phospho-specific antibodies with the power of flow cytometry to enhance phospho protein study. In our assay, cells are stimulated by cytokine, fixed, and permeabilized. They are then stained with fluorescently conjugated antibodies to surface markers to identify specific cell populations and fluorescently conjugated phospho-specific antibodies. 90^{th} percentile of each phospho-specific antibody from stimulated cells is compared to that of unstimulated cells to quantitate the level of phosphorylation of each protein in response to stimulation. Comparing the level of phosphorylation between samples can offer insight to the status of the immune system.

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
2.4. Cytokine aliquots (IFNα, IFNγ, IL-6, IL-7, IL-10, IL-21, IL-2)
2.5. 16% PFA
2.6. Methanol
2.7. FACS buffer (PBS with 2% FBS and 0.1% Na Azide)
2.8. Brilliant Stain Buffer (BD catalogue# 563794)
2.9. Phenotyping and phosphoprotein antibodies
2.10. 37°C water bath
2.11. Biosafety cabinet
2.12. Centrifuge
2.13. CO_2 incubator at 37°C
2.14. Calibrated pipettes

3. Procedure

Thaw PBMC

3.3. Warm media to 37°C in water bath. Each sample will require 22ml of media (complete RPMI) 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. Run one control PBMC with each batch of samples.

3.4. Remove samples from liquid nitrogen and transport to lab on dry ice.

3.5. Place 10ml of warmed benzonase media into a 15ml tube, making a separate tube for each sample.

3.6. Thaw frozen vials in 37°C water bath.

3.7. When cells are nearly completely thawed, carry to hood.

3.8. 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.

3.9. Continue with the rest of the samples as quickly as possible.

3.10. Centrifuge cells at 1650 rpm (RCF=545) for 8 minutes at room temperature.

3.11. Remove supernatant from the cells and resuspend the pellet by tapping the tube.

3.12. Gently resuspend the pellet in 1ml warmed benzonase media. Filter cells through a 70 micron cell strainer if needed. Add 9ml more warmed benzonase media to the tube.

3.13. Centrifuge cells at 1650 rpm (RCF=545) for 10 minutes at room temperature. Aspirate supernatant from the cells.

3.14. Resuspend the pellet in 1ml warm benzonase free media (complete RPMI).

3.15. Count cells with Vicell (or hemocytometer if necessary). To count, take 20 µl cells and dilute with 480ul PBS in vicell counting chamber. Load onto Vicell as PBMC with a 1:25 dilution factor.

3.16. Adjust the cell concentration to 5* 10^6 cells/ml with warm media (complete RPMI wihtout benzonase). Formula=Vicell count divided by 5.

3.17. Using a multichannel pipette, add 100 µl cells (0.5 * 10^6 cells) into each of eight wells of a shallow (round bottom) 96-well plate.

3.18. Rest for another 2 hours at 37°C in CO_2 incubator. During rest period, prepare the stimulation plate.

Example of a full plate:

1	2	3	4	5	6	7	8	9	10	11	12
unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim
IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa
IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg
IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6
IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7
IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10
IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21
IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2

Stimulate cells

3.19. Prepare cytokines in plain RPMI with enough volume to pipette 25 µl into a well for each sample and control. See chart below for dilution for a full plate.

3.20. Prepare enough cytokine into one row of a fresh deep well block to pipette. Remember to add plain media to the well for unstimulated wells.

3.21. Example of cytokine dilutions in a deep well plate:

	stock conc.	10^6 U/ml	100 ug/ml	100 ug/ml	100 ug/ml	100 ug/ml	100 ug/ml	100 ug/ml
	Final conc.	10^3 U/ml	50 ng/ml	50 ng/ml	50 ng/ml	50 ng/ml	50 ng/ml	50 ng/ml
	1	2	3	4	5	6	7	8
A	**unstim**	**IFNa**	**IFNg**	**IL-6**	**IL-7**	**IL-10**	**IL-21**	**IL-2**
	RPMI media	1:200	1:400	1:400	1:400	1:400	1:400	1:400
dilution (µl)	1000	5 + 995	2.5 +997.5	2.5 +997.5	2.5 +997.5	2.5 +997.5	2.5 +997.5	2.5 +997.5

3.22. Remove rested cells in the 96-well shallow plate from incubator and stimulate by adding 25 µl of each cytokine

with multichannel plate to each row of patient samples. Change tips between each patient. Work as rapidly as possible.

3.23. Tap plate to mix, and incubate 15 minutes at 37°C in CO_2 incubator.
3.24. Remove cells from incubator at the appropriate timepoint and using a multichannel pipette, add 20 µl 16% PFA to each row of patient samples in the 96-well shallow plate. Pipette up and down to mix for each patient. Change tips between patients. Add PFA in the same order that you added the cytokine stimulation.
3.25. Incubate 10 minutes at room temperature.
3.26. Add 100 µl FACS buffer to each well of the deep well block.
3.27. Centrifuge cells at 1800 rpm for 8 minutes at 4 °C.
3.28. Wash 2x in FACS buffer.
3.29. Aspirate the supernatant slowly and gently between washes.
3.30. Permeabilize the cells by adding 200µl cold MeOH to each well of the deep well block using a multichannel pipette. Pipette up and down to mix for each patient. Change tips between patients.
3.31. Incubate at least 20 minutes on ice. Cells can be stored at this point at -80°C.
3.32. Wash 2x in FACS buffer.
3.33. Centrifuge cells at 2000 rpm for 8 minutes at 4 °C.
3.34. Aspirate the supernatant slowly and gently between washes.
3.35. Resuspend pellets in residual buffer.
3.36. Prepare the staining cocktail in a fresh shallow 96-well plate according to calculations as in the table below.

Antibody staining panel	Fluorochrome	µl / sample	X # of samples	Total µl
CD3	BV421	2.5	10	25
CD4	PerCP-Cy5.5	20	10	200
CD20	PerCp-Cy5.5	10	10	100
CD33	PE-Cy7	2.5	10	25
CD45RA	BV510	2.5	10	25
pSTAT-1	Ax488	10	10	100
pSTAT-3	Ax647	10	10	100
pSTAT-5	PE	10	10	100
Brilliant Stain Buffer	-	50	10	500

3.37. Add 20 µl volume of staining cocktail to each sample.
3.38. Add appropriate amount of single antibodies to beads for compensation controls.
3.39. Incubate for 30 minutes at room temperature, cover with a foil during this step.
3.40. Wash 2X in FACS buffer.
3.41. Centrifuge cells at 2000rpm for 8 minutes at 4 °C.
3.42. Aspirate the supernatant slowly and gently between washes.
3.43. Resuspend in 200 µl FACS buffer.
3.44. Acquire data on LSRII using defined protocol.