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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. MFI 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. Pacific Orange, frozen aliquot (Invitrogen cat#P30253)
2.8. Alexa 750 frozen aliquot (Invitrogen cat#A20011)
2.9. FACS buffer (PBS with 2% FBS and 0.1% Na Azide)
2.10. Phenotyping and phosphoprotein antibodies
2.11. 37°C water bath
2.12. Biosafety cabinet
2.13. Centrifuge

2.14. CO_2 incubator at 37°C
2.15. Calibrated pipettes

3. Procedure

Thaw PBMC

3.3. 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 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 1400 rpm (RCF=390) 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 1400 rpm (RCF=390) for 8 minutes at room temperature. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
3.14. Resuspend cells in 1ml warm benzonase media.

3.15. Count cells with Vicell (or hemocytometer if necessary). 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.

3.16. Adjust the cell concentration to 2.5 * 10^6 cells/ml with warm media (no more benzonase at this point.) Formula=Vicell count divided by 2.5)

3.17. Using a multichannel pipette, add 200 µl cells (0.5 * 10^6 cells) into each of eight wells of a 96-well deep well plate. Add four extra aliquots of cells (any donor) to the upper right of the plate to be used as compensation controls for the barcoding.

3.18. Rest for another 1 hour- 1.5 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		us
IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa		us
IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg		PO
IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6		Ax
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-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		

Stimulate cells

3.19. Prepare cytokines at 5X concentrations, with enough volume to pipette 50µl into a well for each sample and control. See chart below for dilution for a full plate.

3.20. Add enough 5x cytokine (600µl for a full plate) into one row of a fresh deep well block to pipette. Remember to add plain media to the well for unstimulated and 2 barcode compensation wells.

Example of cytokine dilutions:

	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.	10^4 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
	media	1:20	1:400	1:400	1:400	1:400	1:400	1:400
dilution1		10 + 190	10 + 390	5+195	5+195	5+195	5+195	5+195
dilution 2		*3	200+1800	200+1800	200+1800	200+1800	200+1800	200+1800
deep well	600	30+570	600	600	600	600	600	600

3.21. Remove rested cells in the deep well block from incubator and stimulate by adding 50µl of 5x cytokines with multichannel plate to each row of patient samples. Change tips between each patient. Work as rapidly as possible.
3.22. Tap plate to mix, and incubate 15 minutes at 37°C in CO_2 incubator.
3.23. Remove cells from incubator at the appropriate timepoint and using a multichannel pipette, add 25µl 16% PFA to each row of patient samples in the deep well block. 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.
3.24. Incubate 10 minutes at room temperature.
3.25. Add 1.2 ml PBS to each well of the deep well block.
3.26. Centrifuge cells at 2000 rpm for 8 minutes at 4 °C.
3.27. Decant supernatant from the cells. Permeabilize the cells by adding 600µl 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.
3.28. Incubate at least 20 minutes on ice. Cells can be stored at this point at -80°C.

Barcode and stain samples

3.29. Dissolve Pacific Orange into 250µl DMSO (0.2µg/µl). This concentration will be for the Hi level of staining. Make an intermediate dilution of this by adding 60µl to 340µl DMSO.

3.30. Dissolve Alexa 750 into 160µl of DMSO (0.31µg/µl), This concentration will be for the Hi level of staining. Make an intermediate dilution of this by adding 45µl to 255µl DMSO. (For a full plate you will need 2 aliquots of Alexa 750 to have enough Hi)

3.31. Prepare barcode plate by adding barcode reagents as follows to first row of a fresh 96-well deep well plate. Using a multichannel pipette, transfer 16µl to appropriate wells of a new deep well plate labeled for patient samples.

Example of barcode plate:

	1	2	3	4	5	6	7	8
DMSO	250	125	125	125	0	125	0	0
PO	0	125 int	**125 hi**	0	125 int	0	125 int	**125 hi**
Ax750	0	0	0	125 int	125 int	**125 hi**	**125 hi**	125 int

To PO comp control, add 8µl PO hi. To Alexa 750 comp control add 8µl Alexa 750 hi. To both add 8µl DMSO.

3.32. Remove samples from freezer.

3.33. Mix each samples row well with multichannel pipette and transfer 600ul to appropriate row in barcode plate.

3.34. After transferring all samples to barcode plate, Add 400µl of cold PBS to all wells of the plate.

3.35. Incubate at 4°C for 30 minutes.

3.36. Wash 2x in FACS buffer.

3.37. Combine all wells from a sample into one FACS tube. Repeat for all samples, each sample going into it's own FACS tube.

3.38. Centrifuge cells at 2000rpm for 8 minutes at 4 °C.

3.39. Decant tubes.

3.40. Resuspend pellets in residual buffer.

3.41. Prepare the staining cocktail according to calculations below.

4.

Staining panel		ul/ sample	X # of samples	Total ul
CD3	Pacific Blue	5	10	50
CD4	PerCP-Cy5.5	20	10	200
CD20	PerCp-Cy5.5	10	10	100
CD33	PE-Cy7	2.5	10	25
CD45RA	Qdot 605	0.25	10	2.5
pSTAT-1	Ax488	10	10	100
pSTAT-3	AX647	10	10	100
pSTAT-5	PE	10	10	100

4.3. Add 65µl volume of staining cocktail to each sample. Add appropriate amount of single antibodies to beads for compensation controls.
4.4. Incubate 30 minutes in refrigerator.
4.5. Wash 2X in FACS buffer.
4.6. Resuspend in 250 ul FACS buffer.
4.7. Acquire data on LSRII using defined protocol.