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# TCR/BCR-Stimulated Phospho-Flow 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 through T cell and B cell receptors, 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. BCR stim (anti-human IgG (BD cat # 555784), anti-human IgM (BD cat # 555780), H2O2)
2.5. TCR stim (DYNABEADS HUMAN T-ACT CD3/CD28, Invitrogen, cat # 11132D)
2.6. Magnet for Dynabead separation (BD I Mag cat # 552311)
2.7. 16% PFA
2.8. Methanol
2.9. Pacific Orange, frozen aliquot (Invitrogen cat #P30253)
2.10. Alexa 750 frozen aliquot (Invitrogen cat #A20011)
2.11. FACS buffer (PBS with 2% FBS and 0.1% Na Azide)
2.12. Phenotyping and phosphoprotein antibodies

2.13. 37°C water bath
2.14. Biosafety cabinet
2.15. Centrifuge
2.16. $CO_2$ incubator at 37°C
2.17. 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 11 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 three wells of a 96-well deep well plate. Add four extra aliquots of cells (any donor) to the bottom 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 tube.

3.19.

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 |
| TCR | TCR | TCR | TCR | TCR | TCR | TCR | TCR | TCR | TCR | TCR | TCR |
| BCR | BCR | BCR | BCR | BCR | BCR | BCR | BCR | BCR | BCR | BCR | BCR |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| US | US | PO | Ax | | | | | | | | |

**Stimulate cells**

3.20. For T cell stimulation pipet 700 ul of DYNABEADS HUMAN T-ACT CD3/CD28 in a 12x 75 tube, add media, place it on the magnet separator for 2 minutes.

3.21. Using a 2 ml pipet remove all the media from the tube, so that most of the media is removed. Now resuspend the beads in 700 ul media.

3.22. For B cell stimulation, prepare media as follows (enough for 1 plate):
1434 µl media
30µl anti-human IgG (final concentration 10µg/ml)
30µl anti-human IgM (final concentration 10µg/ml)
6µl 3% H2O2.

3.23. Remove rested cells in the deep well block from incubator

3.24. Add 50µl of media to the first row (unstimulated)

3.25. Stimulate by adding 50µl of TCR stim with pipette to the second row of patient samples. Change tips between each patient. Work as rapidly as possible.

3.26. Tap plate to mix, and incubate 30 minutes at 37°C in $CO_2$ incubator.

3.27. After 26 min add 100µl of B cell stimulation media to the third row.

3.28. Remove plate from incubator at the appropriate timepoint ( 4 minutes) 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 BCR stim and then the TCR stim.

3.29. Incubate 10 minutes at room temperature.

3.30. Add 1.2 ml PBS to each well of the deep well block.

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

3.32. 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.

**3.33.** Incubate at least 20 minutes on ice.

## 4. Barcode and stain samples

4.1. Dissolve Pacific Orange into 250µl DMSO (0.2µg/µl). This concentration will be for the Hi level of staining.

4.2. Dissolve Alexa 750 into 160µl of DMSO (0.31µg/µl). This concentration will be for the Hi level of staining.

4.3. 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:

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO |
| PO | PO | PO | PO | PO | PO | PO | PO | PO | PO | PO | PO |
| Ax | Ax | Ax | Ax | Ax | Ax | Ax | Ax | Ax | Ax | Ax | Ax |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |

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

4.4. Remove samples from freezer.

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

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

4.7. Incubate at 4°C for 30 minutes.

4.8. Wash 2x in FACS buffer.

4.9. Combine all 3 wells from a sample into one FACS tube. Repeat for all samples, each sample going into it's own FACS tube.

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

4.11. Decant tubes.

4.12. Resuspend pellets in residual buffer.

4.13. Prepare the staining cocktail according to calculations below.

5.

| Staining panel | | ul/ sample | X # of samples | Total ul | |
|---|---|---|---|---|---|
| CD3 | Pacific Blue | 5 | 12 | 60 | BD 558117 |
| CD4 | PerCP-Cy5.5 | 20 | | 240 | BD 341654 |
| CD20 | PerCp-Cy5.5 | 10 | | 120 | BD 558021 |
| CD33 | PE-Cy7 | 2.5 | | 30 | BD 333946 |
| CD45RA | Qdot 605 | 0.25 | | 3 | Invitrogen Q10047 |
| P38 | Ax488 / FITC | 2 | | 24 | CST # 4551S |
| pERK1/2 | Ax647 / APC | 2 | | 24 | CST #4284S |
| $pPLC_{gamma2}$ | PE | 10 | | 120 | BD 558490 |

5.7. Add 45µl volume of staining cocktail to each sample. Add appropriate amount of single antibodies to beads for compensation controls.
5.8. Incubate 30 minutes in refrigerator.
5.9. Wash 2X in FACS buffer.
5.10. Resuspend in 250 ul FACS buffer.
5.11. Acquire data on LSRII using defined protocol.