**Stimulating Frozen PBMCs for CyTOF Analysis**

1. Prepare complete media (cRPMI)

* RPMI 1640 (+ L-glutamine) supplemented with 25mM HEPES and 10% FBS

1. Prepare thawing media (tRPMI)

* cRPMI supplemented with 40µg/mL sodium heparin, 0.025U/mL (1:10,000) benzonase, and 100U/mL penicillin + 100µg/mL streptomycin (1:100)

1. Warm cRPMI, tRPMI, and non-supplemented RPMI in a 37°C water bath or incubator
2. Thaw frozen (liquid nitrogen) PBMC cryotube by swirling in a 37°C water bath until a small chunk of ice remains

**Note:** if PBMC vial was submerged in liquid nitrogen (instead of being kept in the vapor phase), great care should be taken when thawing the sample, as any liquid nitrogen that may have seeped into the tube can erupt when the liquid boils at room temperature

1. Using an electronic pipettor with a 10mL disposable pipette, aspirate 9mL warmed tRPMI and add to PBMC tube dropwise until sample is completely thawed, then aspirate entire sample back into pipette (~10mL total volume)
2. Transfer thawed sample to a 15mL conical tube and gently invert 5 times to mix
3. Store in a 37°C water bath or incubator until ready to spin
4. Repeat steps 4-7 for all samples
5. Centrifuge thawed PBMC samples at 300xg and RT for 15 minutes
6. Aspirate supernatant and gently flick bottom of tube to loosen cell pellet (DO NOT vortex!!!)
7. Add 10mL cRPMI to each sample and gently invert 5 times to mix
8. Take 10µL of each sample for counting
9. Loosen caps on tubes and allow PBMCs to recover for 1-2 hours in a 37°C water bath or incubator
10. Count cells during recovery period
11. Centrifuge recovered PBMCs at 300xg and RT for 15 minutes
12. Aspirate supernatant
13. Gently flick bottom of tube to loosen cell pellet (DO NOT vortex!!!)
14. Add 10mL room temperature DPBS and invert tube 5 times to mix
15. Repeat for all samples
16. Centrifuge tubes at 300xg and RT for 15 minutes
17. Aspirate supernatant
18. Gently flick bottom of tube to loosen cell pellet (DO NOT vortex!!!) and re-suspend cells in appropriate volume of warmed non-supplemented RPMI to achieve desired concentration
19. Repeat for all samples
20. Keep PBMCs in a 37°C water bath or incubator until ready to stimulate
21. Dispense stimulants into FACS tubes:
    1. Unstimulated control: 5µL DPBS (or volume equivalent to stimulations)
    2. Stimulation: 5µL 10µg/mL Cytokine or 5µL 100µg/mL LPS
22. Add 500µL PBMCs to FACS tube and gently pipette up and down 3 times to mix. Volumes of stimulants and cells can be adjusted according to experimental design.
23. Repeat for all conditions

**Note:** where possible, it is best to add cells to all conditions simultaneously, e.g. with a multi-channel pipettor. This will minimize the difference in timing between conditions.

1. Incubate FACS tubes in a 37°C water bath for 15 minutes (an incubator is not recommended for this step)
2. Add 700µL proteomic stabilizer to each FACS tube and mix thoroughly by pipetting up and down 3 times. Note that the proper ratio of proteomic stabilizer to cell suspension is 1.4mL stabilizer per 1mL of cell suspension. Adjust accordingly where necessary.

**Note:** where possible, it is best to add stabilizer to each FACS tube simultaneously, e.g. with a multi-channel pipettor

1. Fix PBMCs at room temperature for 15 minutes
2. Immediately freeze fixed PBMCs in 2mL cryovials at -80°C

**Note:** if cells cannot be frozen immediately, they may be stored on wet ice for up to 15 minutes prior to placing them in the freezer