Staining Protocol for Flow Cytometry of Human PBMCs: Immune Subset Changes and Peripheral Immunoscore Analysis

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| 1. | Warm water bath to 37C and warm media (IMDM with 10% FBS) to 37C. |  |
| 2. | Label 15 ml conical tubes. |  |
| 3. | Retrieve PBMC from liquid nitrogen; keep on dry ice until use. |  |

Do the following steps rapidly to ensure high viability (only thaw a few samples at a time)

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| 4. | Thaw samples rapidly in water bath until just thawed (still icy). |  |
| 5. | Wipe with paper towel sprayed with ethanol. |  |
| 6. | Add 1ml PBMCs to the conical. |  |
| 7. | Add 1ml warm media, 1 drop at a time, to the conical; then, fill to 10ml with warm media. |  |
| 8. | Spin at 800xg for 5 min at room temperature (RT). |  |
| 9. | Discard supernatant and resuspend pellet with 1ml warm media, then fill to 10ml. |  |
| 10. | Spin at 800xg for 5 min at RT. |  |
| 11. | Discard supernatant and resuspend pellet with 1ml warm media, then fill to 10ml. |  |
| 12. | Spin at 800xg for 5 min at room temperature (RT). |  |
| 13. | Count cells, then resuspend at 5x106/ml in media. |  |
| 14. | Transfer 200ul (1x106 cells) to a 96 well U-bottom plate for staining. |  |
| 15. | Spin at 800xg for 5 min at room temperature, and then decant supernatant. |  |
| 16. | Resuspend cells in 200ul/well PBS. Centrifuge at 800xg for 5 min at 4C. (Cells can now be kept on ice) Discard supernatant. |  |

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| 17. | Prepare master mix containing live/dead discriminator (see antibody panel)   |  |  |  |  | | --- | --- | --- | --- | | Reagent | # wells | Volume/well | Volume for x wells | | Live/Dead |  | 0.1ul |  | | PBS |  |  |  | |  |
| 18. | Add 100ul/well live/dead discriminator master mix to appropriate wells. Add 100ul/well PBS to the unstained control (USC). |  |
| 19. | Cover plate with foil and incubate plate on ice for 15 minutes. |  |
| 20. | Spin at 800xg for 5 min at 4C. Discard supernatant. |  |
| 21. | Add 200ul/well PBS with 1% BSA, mix with pipette to resuspend pellet. |  |
| 22. | Spin at 800xg for 5 min at 4C. Discard supernatant. |  |
| 23. | Add 100ul/well PBS with 1% BSA plus FC block. Incubate on ice for 5 min.   |  |  |  |  | | --- | --- | --- | --- | | Reagent | # wells | Volume/well | Volume for x wells | | FC Block |  | 2ul |  | | PBS with 1% BSA |  |  |  | |  |
| 24. | Add the extracellular antibodies and mix gently with pipette\*. |  |
| 25. | Cover plate with foil and incubate on ice for 20 min. |  |
| 26. | Add 100ul/well of PBS with 1% BSA. |  |
| 27. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 28. | Resuspend in 200ul/well PBS with 1% BSA. |  |
| 29. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 30. | Resuspend in 100ul/well of undiluted Cytofix/Cytoperm. |  |
| 31. | Cover plate with foil and incubate at RT for 20 min. |  |
| 32. | Prepare Perm/Wash Buffer (PWB). Dilute 1:10 in dH2O. |  |
| 33. | Add 100ul/well PWB. |  |
| 34. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 35. | Resuspend in 200ul/well PWB. |  |
| 36. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 37. | Make master mix for intracellular antibodies.  See attached table for lot # and volume. |  |
| 38. | Add 100ul/well PWB. |  |
| 39. | Add intracellular antibodies to appropriate wells, resuspend pellet. |  |
| 40. | Cover plate with foil and incubate at RT for 20 min. |  |
| 41. | Add 100ul/well PWB. |  |
| 42. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 43. | Resuspend in 200ul/well PWB. |  |
| 44. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 45. | Dilute Cytofix 1:2 in dH2O. |  |
| 46. | Resuspend PBMC in 200ul/well Cytofix. |  |
| 47. | Cover plate with foil and incubate at 4C for ≥30 min. (Max 48hrs)  Time/Date in Cytofix: Time/Date switch to PBS: |  |
| 48. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 49. | Resuspend in 200ul/well PBS. Run on cytometer. |  |

\*Note: The antibodies must be tested prior to performing staining by:

1. Titration (E.g. if manufacturer recommends 5 ul/test, titrate 10, 5, 2, 1 and 0.5 ul/test)

2. Testing of compatibility with other antibodies in the panel (using single color staining and FMO- fluorescence minus-one)

3. Gates based on FMO

Materials and Reagents:

-IMDM

-Fetal bovine serum

-PBS CA-free Mg-free

-PBS with 1% BSA

-Anti-human antibodies and live/dead discriminator (see panels)

-eBioscience Fixation/Permeabilization Concentrate (cat # 00-5123-43)

-eBiosceicne Fixation/Permeabilization Diluent (cat # 00-5223-56)

-eBioscience Permeabilization Buffer (10x) (cat # 00-8333-56)

-BioLegend Human TruStain FcX (Fc Receptor Blockign Solution ) (cat# 422302)