Assay Setup:

Day -1: Thaw and Rest PBMCs Date:

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| 1. | Thaw samples rapidly in water bath until just thawed (still icy) |  |
| 2. | Wipe with paper towel sprayed with ethanol |  |
| 3. | Add 1ml PBMCs 1 drop at a time to 15ml conical |  |
| 4. | Add 1ml warm media 1 drop at a time to 15ml conical, then add warm media up to 10ml |  |
| 5. | Spin at 2000rpm (800xg) for 5 min at room temperature (RT) |  |
| 6. | Discard supernatant, resuspend pellet in 10ml warm media |  |
| 7. | Spin at 2000rpm (800xg) for 5 min at RT |  |
| 8. | Discard supernatant, resuspend pellet in 1ml complete media |  |
| 9. | Incubate **overnight** (O/N) at 37C, 5% CO2 in a sterile 6 well plate (1ml cells + 4ml complete media)  Time in: |  |

Day 0: *In vitro* Stimulation Date:

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| 10. | Gently scrape cells, wash 1x with complete media.  Date: Time out: |  |
| 11. | Count and seed 2-2.5x10^6cells/ml in a sterile 12 well dish (1 ml per well) |  |
| 12. | Dilute peptides.  Add 40ul DMSO to vial that contains 25ug lyophilized peptides (0.625ug/ul)  Prepare 1:10 dilution in media (0.0625ug/ul)  Add 1.6ul per 1ml of media+cells of 1:10 dilution to each well (final concentration 0.1ug/mL) |  |
| 13. | Add peptides to PBMCs (peptides will be in wells for the entire duration of stimulation) |  |
| 14. | Swirl plate to gently mix or gently pipette. |  |
| 15. | Incubate at 37C 5% CO2  Time in: |  |

Day 3: Addition of Cytokines Date:

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| 16. | Add 1ml fresh complete media containing IL-7 and IL-15 at a final concentration of 10ng/ml. Place in 37C incubator 5% CO2. Calculations:  \_\_\_\_\_\_\_ wells x 2 x 0.2 = \_\_\_\_\_\_\_ ml of 10x solution;  Use \_\_\_\_\_\_ aliquots of IL-7 and \_\_\_\_\_\_ aliquots of IL-15 (each aliquot = 20 ml)  (Find aliquots at -80C)  save \_\_\_\_\_\_\_ ml (1/2 of 10x solution) for day 5  \_\_\_\_\_\_\_ ml (1/2 of 10x solution) + \_\_\_\_\_\_\_\_ ml media for 2x solution  Time in: |  |

Day 5: Addition of Cytokines Date:

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| 17. | Remove 1ml of supernatant from well and save at -20C. |  |
| 18. | Add \_\_\_\_\_\_ mL media to the 10x solution of IL-7 and IL-15 made on Day 3. Add 1ml/well for a final concentration of 10ng/ml IL-7 and IL-15. Place in 37C incubator.  Time in: |  |

Day 7: Rest Cells Date:

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| 19. | Remove 1ml of supernatant from well and save at -20C. |  |
| 20. | Collect non-adherent cells, transfer to conical. |  |
| 21. | Rinse well with media, and transfer to same conical as non-adherent cells. |  |
| 22. | Add 1ml complete media to well to prevent drying. |  |
| 23. | Spin non-adherent cells for 5 min, remove supernatant, and resuspend in 1ml media. |  |
| 24. | Transfer non-adherent cells to the same well that the sample was removed from. Final well volume=2ml. |  |
| 25. | Allow cells to rest in 37C incubator for **4 days**.  Time in: |  |

Day 11: Restimulate Cells Date:

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| 26. | Collect non-adherent cells, then gently scrape well to collect adherent cells. |  |
| 27. | Combine adherent and non-adherent cells and wash 1x with complete media. |  |
| 28. | Count cells and adjust concentration to ~1x10^6/well in 200ul IMDM |  |
| 29. | Transfer cells to 96 well round bottom plate. Each well should contain ~200ul. |  |
| 30. | Add peptide to each well.  Add 40ul DMSO to vial that contains 25ug lyophilized peptides (0.625ug/ul)  Prepare 1:100 dilution in media (0.00625ug/ul)  Add 3.2ul per 200ul of media+cells of 1:100 dilution to each well (final concentration 0.1ug/mL) |  |
| 31. | Add 5ul/well CD107a. |  |
| 32. | Allow cells to incubate at 37C for **2 hours**.  Time in: Time out: |  |
| 33. | Prepare Golgi Plug and Golgi Stop master mix and add 5ul/well.  (Find Golgi Plug and Stop at 4C)  Prepare for \_\_\_\_\_\_\_\_\_\_ wells.   |  |  |  | | --- | --- | --- | | Reagent | Volume/well | Volume for \_\_\_\_\_\_\_ # of wells | | IMDM | 4.66ul |  | | Golgi Stop (Monensin) | 0.14ul |  | | Golgi Plug (Brefeldin) | 0.20ul |  | |  |
| 34. | Mix by gently pipetting and incubate for **22 additional hours** at 37C, 5% CO2. Time in: |  |

Day 12: Intracellular Cytokine Staining Date:

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| 35. | After \_\_\_\_\_\_\_ hour incubation (\_\_\_\_\_ hours with Golgi Plug/ Golgi Stop) remove plates from the incubator. Time out: |  |
| 36. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 37. | Resuspend in 200ul/well PBS. |  |
| 38. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 39. | Prepare master mix containing live/dead discriminator (see antibody panel)   |  |  |  |  | | --- | --- | --- | --- | | Reagent | # wells | Volume/well | Volume for x wells | | Live/Dead |  | 0.1ul |  | | PBS |  |  |  | |  |
| 40. | Add 100ul/well live/dead discriminator master mix to appropriate wells. Add 100ul/well PBS to the unstained control (USC). |  |
| 41. | Cover plate with foil and incubate plate on ice for **15 minutes**. |  |
| 42. | Spin at 800xg for 5 min at 4C. Discard supernatant. |  |
| 43. | Add 200ul/well PBS with 1% BSA, mix with pipette to resuspend pellet. |  |
| 44. | Spin at 800xg for 5 min at 4C. Discard supernatant. |  |
| 45. | Add 100ul/well PBS with 1% BSA plus FC block. Incubate 5 minutes on ice.   |  |  |  |  | | --- | --- | --- | --- | | Reagent | # wells | Volume/well | Volume for x wells | | FC Block |  | 2ul |  | | PBS with 1% BSA |  |  |  | |  |
| 46. | Add the extracellular antibodies and mix gently with pipette\*. |  |
| 47. | Cover plate with foil and incubate on ice for **20 min**. |  |
| 48. | Add 100ul/well of PBS with 1% BSA. |  |
| 49. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 50. | Resuspend in 200ul/well PBS with 1% BSA. |  |
| 51. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 52. | Resuspend in 100ul/well of undiluted Cytofix/Cytoperm. |  |
| 53. | Cover plate with foil and incubate at RT for **20 min**. |  |
| 54. | Prepare Perm/Wash Buffer (PWB). Dilute 1:10 in dH2O. |  |
| 55. | Add 100ul/well PWB. |  |
| 56. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 57. | Resuspend in 200ul/well PWB. |  |
| 58. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 59. | Make master mix for intracellular antibodies.  See attached table for lot # and volume. |  |
| 60. | Add 100ul/well PWB. |  |
| 61. | Add intracellular antibodies to appropriate wells, resuspend pellet. |  |
| 62. | Cover plate with foil and incubate at RT for **20 min**. |  |
| 63. | Add 100ul/well PWB. |  |
| 64. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 65. | Resuspend in 200ul/well PWB. |  |
| 66. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 67. | Dilute Cytofix 1:2 in dH2O. |  |
| 68. | Resuspend PBMC in 200ul/well Cytofix. |  |
| 69. | Cover plate with foil and incubate at 4C for **≥30 min**. (Max 48hrs)  Time/Date in Cytofix: Time/Date switch to PBS: |  |
| 70. | Spin at 800xg for 5 min at RT. Discard supernatant. |  |
| 71. | Resuspend in 200ul/well PBS. Run on cytometer. |  |