**Cell Preparation, Stimulation, and Plating for U01 Influenza Cytokine and Chemokine Multiplex Assay**

Reagents

* Thawing media with DNase
* Culture media
* H1N1 virus

1. Check out vials from VATS.
2. Warm RPMI thawing media in 37°C water bath for 15 minutes.
3. Label one 15ml conical tube with subject ID for each subject.
4. Add 1ml thawing media to each 15ml conical tube.
5. Thaw PBMCs in 37°C water bath until small chunks of ice remain.
6. Uncap cryovials and transfer PBMCs to designated 15ml conical tubes.
7. Add thawing media slowly, doubling the volume until a final volume of 10ml is reached.
8. Cap each tube and invert 5 times to mix.
9. Centrifuge at 1200 rpm at room temperature for 7 minutes.
10. Remove supernatant by vacuum aspiration, leaving only a few hundred µl in the tube. Do not aspirate the cell pellet.
11. Resuspend the cells in 10ml thawing media.
12. Cap each tube and invert 5 times to mix.
13. Place in 37°C water bath for 20 minutes. Invert tubes to mix at 10 minutes.
14. During incubation, prepare FACS tubes for counting. 200µl of 1X PBS and 37.5µl of trypan blue.
15. Cool cells on ice for 7 minutes.
16. Centrifuge at 1200 rpm at 4°C for 7 minutes.
17. Remove supernatant by vacuum aspiration without disturbing the cell pellet.
18. Resuspend cells in each tube in 2ml culture media.
19. Filter if needed.
20. Mix cells by inverting and add 12.5µl to the appropriate FACS tube for counting.
21. Mix FACS tube by vortexing and add 10µl to the hemocytometer.
22. Place hemocytometer under microscope and count cells.
23. Record results in excel worksheet.
24. Add the indicated amount of culture media to the appropriate conical tube to adjust the cell concentration to 2 X 106 cells/ml. The minimum number of cells for all wells are 1.2 X 106. If less than 1 X 106, thaw another vial.
25. Place 100µl of cells from the 2 subjects with the highest cell counts (do not mix cells from subjects) in wells H9 and H10.
26. Place 100µl of cells in each well of a 96 well round bottom plate as shown below.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A |  |  | Subj. 1 Unstim | Subj. 1 Unstim | Subj. 1 Unstim | Subj. 1 Stim | Subj. 1 Stim | Subj. 1 Stim | Subj. 9 Unstim | Subj. 10 Unstim | Subj. 11 Unstim | Subj. 12 Unstim |
| B |  |  | Subj. 2 Unstim | Subj. 2 Unstim | Subj. 2 Unstim | Subj. 2 Stim | Subj. 2 Stim | Subj. 2 Stim | Subj. 9 Unstim | Subj. 10 Unstim | Subj. 11 Unstim | Subj. 12 Unstim |
| C |  |  | Subj. 3 Unstim | Subj. 3 Unstim | Subj. 3 Unstim | Subj. 3 Stim | Subj. 3 Stim | Subj. 3 Stim | Subj. 9 Unstim | Subj. 10 Unstim | Subj. 11 Unstim | Subj. 12 Unstim |
| D |  |  | Subj. 4 Unstim | Subj. 4 Unstim | Subj. 4 Unstim | Subj. 4 Stim | Subj. 4 Stim | Subj. 4 Stim | Subj. 9 Stim | Subj. 10 Stim | Subj. 11 Stim | Subj. 12 Stim |
| E |  |  | Subj. 5 Unstim | Subj. 5 Unstim | Subj. 5 Unstim | Subj. 5 Stim | Subj. 5 Stim | Subj. 5 Stim | Subj. 9 Stim | Subj. 10 Stim | Subj. 11 Stim | Subj. 12 Stim |
| F |  |  | Subj. 6 Unstim | Subj. 6 Unstim | Subj. 6 Unstim | Subj. 6 Stim | Subj. 6 Stim | Subj. 6 Stim | Subj. 9 Stim | Subj. 10 Stim |  |  |
| G |  |  | Subj. 7 Unstim | Subj. 7 Unstim | Subj. 7 Unstim | Subj. 7 Stim | Subj. 7 Stim | Subj. 7 Stim | Subj. 11 Stim | Subj. 12 Stim |  |  |
| H |  |  | Subj. 8 Unstim | Subj. 8 Unstim | Subj. 8 Unstim | Subj. 8 Stim | Subj. 8 Stim | Subj. 8 Stim | PHA | PHA |  |  |

1. Save any extra cells for other testing.
2. Set up antigen. Mix 800µl virus with 3200µl culture media (per plate).
3. Make PHA by adding 50µl PHA to 950µl culture media.
4. Place 100µl antigen in each well (red in above diagram).
5. Add 100µl medium to unstimulated wells.
6. Add 100µl PHA to the wells H9 and H10.
7. Incubate at 37°C for 48 hours.
8. Fill out U01 Cytokine Assay Plate Map worksheet.
9. At 48 hour harvest, transfer 60µl of supernatant to a new round bottom plate. Place the aliquots in the same position as the original. Transfer 120µl to another round bottom plate, again in the same position. Place all 3 plates in the -80°C freezer.