Protocol 1 – Lyse no Wash

Task 1:

1. Label one 5 ml tube: LNW.
2. Pipette 5 µl of each antibody reagent to the bottom of the tube. (CD3; CD4; CD8; CD19; CD45).
3. Pipette 50 µl of well-mixed, anticoagulated whole blood into the bottom of the tube.
4. Vortex gently to mix. Incubate for 15 minutes in dark at room temperature.
5. Add 450 µl of 1X BD FACS lysing solution to the tube (900 µl DI + 100 µl 10X BD FACS lysing solution). Pipette through several times and incubate for 15 minutes at room temperature in dark.
6. Acquire the sample on FACSLyric with LyseNoWash assay.

Task 2:

1. Repeat steps 1 – 4 from task 1.
2. Add 2 ml of 1X BD FACS lysing solution to the tube. Incubate for 15 minutes at room temperature in dark.
3. Centrifuge the sample at 500 X g for 5 minutes.
4. Decant supernatant and add 0.5 ml of PBS for flow cytometry analysis.
5. Vortex gently and acquire the sample on FACSLyric with LyseNoWash assay.

Protocol 2 – Pharm Lyse™ (Ammonium chloride-based lysis)

Task:

1. Label one 5 ml tube: Pharm Lyse.
2. Pipette 100 µl of well-mixed, anticoagulated whole blood into the bottom of the tube.
3. Pipette 5 µl of each antibody reagent to the tube. (CD3; CD4; CD8; CD19; CD45).
4. Vortex gently to mix. Incubate for 15 minutes in dark at room temperature.
5. Add 2.0 ml of 1X Pharm lysing solution (9 ml DI + 1 ml Pharm lysing solution) to the tube containing up to 200 µl of a whole blood plus monoclonal antibody mixture.
6. Gently vortex each tube immediately after adding the lysing solution.
7. Incubate at room temperature, protected from light, for 15 minutes.
8. Centrifuge 500 X g for 5 minutes.
9. Decant the supernatant, without disturbing pellet.
10. Resuspend the pellet in 0.5 ml PBS-BSA for flow cytometric analysis.
11. Acquire the sample on FACSLyric with PharmLyse assay.

Protocol 3 – Freezing cells in Glycerol for future staining and acquisition.

Task:

1. Label three 15 ml tubes and one 5 ml tube: Glycerol.
2. In one 15 ml tube dilute whole peripheral blood 1:1 with PBS. (3 ml of blood + 3 ml of PBS)
3. Pipette 3 ml of Ficoll Paque to the second 15 ml tube.
4. Carefully overlay 6 ml of diluted blood over the Ficoll solution.
5. Centrifuge the tube at 1200 X g for 20 minutes. With acceleration 1 and 0 brake.
6. Collect the PBMC from the border of Ficoll and plasma to the third 15 ml tube.
7. Wash once in the third 15 ml tube with 10 ml of PBS. And decant supernatant.
8. Take the 5 ml tube and add 100 µl of PBMC and add 11 µl of 16% paraformaldehyde.
9. Incubate for 10 minutes at room temperature.
10. Add 3 ml of PBS and centrifuge at 500 X g for 5 minutes.
11. Decant the supernatant and add 1 ml of FBS + 10% glycerol. (900 µl FBS + 100 µl glycerol)
12. Cap the tube and put it to the freezer at -20.
13. Take from the freezer one 5 ml tube with frozen sample. Let it warm up to the room temperature and then add 3 ml of PBS.
14. Centrifuge the tube for 5 minutes at 500 X g.
15. Decant the supernatant and add 5 µl of all antibody reagents (CD3; CD4; CD8; CD19; CD45).
16. Vortex gently to mix. Incubate for 15 minutes in dark at room temperature.
17. Add 3 ml of PBS and centrifuge at 500 X g for 5 minutes.
18. Decant the supernatant, without disturbing pellet.
19. Resuspend the pellet in 0.5 ml PBS-BSA for flow cytometric analysis.
20. Acquire the sample on FACSLyric with Glycerol assay.

Protocol 4 – Intracellular cytokines detection.

Task:

1. Take two 5 ml tubes and label them: Stimulated and Unstimulated
2. Let the samples warm up to the room temperature and pipette them into the corresponding tube.
3. Add 3 ml of PBS + 0,5% BSA and centrifuge the tubes for 5 minutes at 500 X g.
4. Decant the supernatant and add 1 ml of 1 X BD FACS Lysing solution.
5. Vortex gently and incubate for 10 minutes at room temperature.
6. Centrifuge for 5 minutes at 500 X g.
7. Decant the supernatant as much as possible (touch the pulp with the top of the tube).
8. Add 0.5 ml of 1 X BD Perm2 reagent. (1000 µl DI + 111 µl BD Perm2 solution).
9. Incubate for 25 minutes at room temperature.
10. Add 3 ml of PBS + 0.5% BSA and centrifuge the samples at 500 X g for 5 minutes.
11. Decant the supernatant and add 5 µl of all the antibody reagents to each tube (CD3; CD4; CD8; CD19; CD45; IFNg; TNFa; pTyr).
12. Vortex gently and incubate for 20 minutes in dark at room temperature.
13. Add 3 ml of PBS + 0.5% BSA and centrifuge at 500 X g for 5 minutes.
14. Decant the supernatant, without disturbing the pellet.
15. Resuspend the pellet in 0.5 ml PBS-BSA for flow cytometric analysis.
16. Acquire the sample on FACSLyric with Cytokine assay.

Protocol 5 – Staining of phospho epitope

Task:

1. Take two 5 ml tubes and label them: Stimulated and Unstimulated
2. Let the samples warm up to the room temperature and pipette them into the corresponding tube.
3. Add 3 ml of PBS + 0,5% BSA and centrifuge the tubes for 5 minutes at 500 X g.
4. Decant the supernatant (the pellet should be as dry as possible) and put the tubes on ice.
5. Add 1 ml of ice-cold BD Phosflow™ Perm Buffer II to the cell pellet during vortexing.
6. Incubate for 30 minutes on ice.
7. Centrifuge the tubes at 500 X g for 5 minutes.
8. Decant the supernatant (the pellet should be as dry as possible) and add 3 ml of PBS + 0.5% BSA and centrifuge the tubes once more at 500 X g for 5 minutes.
9. Decant the supernatant and add 5 µl of all reagents to each tube (CD3; CD4; CD8; CD19; CD45; IFNg; TNFa; pTyr).
10. Incubate the samples for 20 minutes at room temperature in dark.
11. Add 3 ml of PBS + 0.5% BSA and centrifuge the samples at 500 X g for 5 minutes.
12. Decant the supernatant, without disturbing the pellet.
13. Resuspend the pellet in 0.5 ml PBS-BSA for flow cytometric analysis.
14. Acquire the sample on FACSLyric with Phospho assay.