

Laboratory Protocols - Ruminant Immunology

Single-Color Immunofluorescence Staining of Sheep Leukocytes

Materials:

1.6M Ammonium Chloride (NH₄Cl)
0.17M Tris-Cl pH 7.2
96-well round-bottom plate
Adhesive "lids"
Phosphate Buffered Saline containing 1% Fetal Calf Serum
Primary antibody (know the isotype) - normally as supernatant
Secondary Antibody (Southern biotech, isotype specific, dilute FITC conjugates with PBS-1%FCS at a ratio of 1:400, dilute PE conjugates with PBS-1%FCS at a ratio of 1:1000)
PBS containing 1% paraformaldehyde
Multichannel Pipettor
Small Tips
P200 and P20 adjustable pipettes.

Methods:

1. For each 10ml of anticoagulated blood, you will need 1 50ml centrifuge tube prepared as follows: 3.6 ml of 1.6M Ammonium Chloride, 32.4ml of distilled water, and 4ml of Tris-Cl pH7.2. Warm to 37C in a water bath.
2. Add 10ml of whole blood to the lysis solution in the 50ml centrifuge tube. Mix gently but thoroughly until the red blood cells lyse (looks like a good red wine). If this takes longer than 2-3 minutes, something is wrong...
3. Centrifuge at 1600 rpm for 7 minutes.
4. Dump off supernatant in the sink. Resuspend cells in 50ml PBS-1% FCS. If you used more than 1 tube, pool all into a single 50ml centrifuge tube
5. Spin the cells at 1600 rpm for 7 minutes.
6. Dump off supernatant in the sink. Resuspend cells in 50ml PBS-1%FCS.
7. Spin the cells at 1600 rpm for 7 minutes.
8. Dump off supernatant in the sink. Resuspend in an appropriate volume (i.e. approximately equal to original blood volume) and count cells. Calculate the TOTAL number of cells you have by using the following formula.

Your count = C; Total volume that you used to resuspend = V

Total cells (T) = Cx10,000xV

Laboratory Protocols - Ruminant Immunology

When finished, add enough PBS-1%FCS to bring to 50ml.

9. Spin the cells at 1600 rpm for 7 minutes.
10. From your cell count in (8), resuspend cells to a final concentration of about 10^8 cells/ml using PBS-1%FCS. The formula to use is:
Your count = T
To calculate volume (in ml) to resuspend your cells, use the formula
Resuspension volume = $T/10^8$
11. Make a template of your staining plate, indicating each antibody to be used. Add 50ul of PBS-1% FCS to the first well of each row to be used. Add 50 ul of primary antibody (normally used undiluted as supernatant). NOTE: NEVER take antibody directly from the stock bottle. Take a 15 ml centrifuge tube, label it, and add 5 ml of antibody to that tube from the stock bottle. Then, use that tube as your source of antibody for the plate. That will allow you to do 100 stainings, and prevents overuse of the stock bottle.
12. Add 50 ul of cells (will be about 5×10^6 cells/well) to each well of the plate.
13. Incubate 10 minutes at 4C (in the refrigerator)
14. Add 75 ul PBS-1% FCS.
15. Spin plate at 1200rpm, 1 minute.
16. In one quick motion, dump the supernatant into the sink. Only invert the plate ONCE.
17. Resuspend each well of cells with 150 ul of PBS-1% FCS.
18. Spin plate at 1200 rpm, 1 minute.
19. In one quick motion, dump the supernatant into the sink. Only invert the plate ONCE.
20. Resuspend each well of cells with 150 ul of PBS-1% FCS.
21. Spin plate at 1200 rpm, 1 minute.
22. In one quick motion, dump the supernatant into the sink. Only invert the plate ONCE.
23. Calculate how much secondary antibody you will need (use the number of wells you are staining, add 2 extras, and multiply that number by 50ul). Dilute the secondary

Laboratory Protocols - Ruminant Immunology

antibody appropriately with PBS-1%FCS (FITC antibodies get diluted 1:400, PE antibodies get diluted 1:1000)

24. Add 50 ul of secondary antibody to each well and resuspend. DON'T carry over between wells.
25. Incubate 10 minutes at 4C (in the fridge).
26. Add 75 ul PBS-1% FCS.
27. Spin plate at 1200rpm, 1 minute.
28. In one quick motion, dump the supernatant into the sink. Only invert the plate ONCE.
29. Resuspend each well of cells with 150 ul of PBS-1% FCS.
30. Spin plate at 1200 rpm, 1 minute.
31. In one quick motion, dump the supernatant into the sink. Only invert the plate ONCE.
32. Resuspend each well of cells with 150 ul of PBS-1% FCS.
33. Spin plate at 1200 rpm, 1 minute.
34. In one quick motion, dump the supernatant into the sink. Only invert the plate ONCE.
35. Resuspend cells in 100 ul PBS-1% Paraformaldehyde. Store in the fridge for up to 1 week.