Fluorescence Activated Cell Sorting of Populations for HIPC IOF CD8/NK miRNA Project

Reagents required

- Thawing medium with DNase
- Flow buffer
- Fluorochrome conjugated antibodies
- FACS tubes

Recipes

Thawing Medium
439.5ml RPM + L-glutamine
50ml FBS
5ml Penicillin-Streptomycin
5ml Sodium Pyruvate
0.5ml DNase
Filter sterilize

Flow buffer
PBS
2% FBS
Filter sterilize

Protocol

All steps should be done in the hood to keep flow buffer sterile.
All centrifugation steps and incubations should be done at 4°C.

1. Identify the subjects' PBMC vials in VATS. Select two PBMC vials with 1ml and one PBMC vial with 500ul. If there are no 500ul vials, then select two 1ml PBMC vials.
2. Create run, find vials, check out vials.
3. Thaw vials briefly in water bath at 37°C.
4. For each subject, transfer volume from the three cryovials to a single 50ml conical tube labeled with subject ID.
5. For each subject, wash the three cryovials with 1ml of 4°C thawing medium and add to the 50ml tube.
6. Bring volume in 50ml tube to 20ml.
7. Centrifuge at 1200 rpm for 5 min at 4°C.
8. Remove media, gently resuspend pellet in 20ml of 4°C thawing medium.
9. Take 12.5ul aliquot out for counting and centrifuge cells again (1200rpm, 5min, 4°C).
10. While cells are spinning, add the 12.5ul aliquot + 12.5ul trypan blue + 75 ul PBS to a FACS tube. Mix and count cells with hemocytometer. Count 4 quadrants and divide the total # of cells in all 4 quadrants by 50. This is the cell concentration x 10^6 per ml. Multiply by 20 to get the total cell #. Calculate volume necessary to resuspend at 2x10^6 cells/ml.
11. After centrifuge is completed, remove media from cells and resuspend at 2x10^6 cells/ml in cold flow buffer. Place cells on ice.
12. Set-up FACS tubes.
    a. Tubes 1-7 for controls. Label tubes #1 - #7.
    b. 2-4 tubes per subject. Label each tube with subject ID.
    c. Place 2.5ml of cells in each subject tube (5x10^6 cells/tube). Make sure that each subject has at least 1 tube.

Table 1. Master Mix Preparation

Reagent	Per tube	Total
Brilliant Dye Staining Solution	50ul	
CD19 Brilliant Violet 421	5ul	
CD4 Brilliant Violet 510	3ul	
CD8 Alexa Fluor 488	3ul	
CD3 PE	20ul	
CD56 Alexa Fluor 647	3ul	
CD16 APC-Cy7	3ul	

a. Combine leftovers and distribute equally among tubes 1-7. You need at least 125ul of cells per tube.

Total	87ul	

13. Centrifuge cells down (1200rpm, 5min, 4°C).
14. Pour off excess volume. ~200ul will be left in tube.
15. Prepare master mix (Table 1) of antibodies and staining buffer in an amber tube. Vortex briefly to mix.
16. Add 87 ul master mix per subject tube.
17. Add 50ul Brilliant Dye Staining Solution to control tubes (#1-#7)
18. Add individual antibodies to control tubes (Table 2).
19. Incubate at 4C for 30 minutes in the dark.
20. Add 4ml flow buffer to each tube.
21. Centrifuge cells down (1200rpm, 5min, 4°C).
22. Add 250ul flow buffer to each control tube.
23. Add 300ul flow buffer to each subject tube.
24. Label a new tube for each sample. Place 70um filter over the tube. Transfer all tubes with stained cells from that sample to the new tube.
25. For every subject prepare 5 additional FACS tubes with 1.5ml of flow buffer solution. Label 4 of the tubes as follows: "B cells"; "CD4 cells"; "CD8 cells"; "NK cells".
26. Place all tubes in bucket on ice.
27. Cover top of ice bucket / tubes in foil and place in cold room until FACS appointment.

Table 2. Staining for Control Tubes

Tube #	Antibody to add	Volume
1	No antibody	-
2	CD19 Brilliant Violet 421	5ul
3	CD4 Brilliant Violet 510	3ul
4	CD8 Alexa Fluor 488	3ul
5	CD3 PE	20ul
6	CD56 Alexa Fluor 647	3ul
7	CD16 APC-Cy7	3ul

Flow Lab

1. Watch technicians set up voltages/compensations.
2. They can pull last experiment up for template. Voltages/Compensations should be very close.
3. Double check that gates to be sorted are the correct cell populations (See Sorting flow-chart on next page).
4. Please ask for post-sort analysis and the sorting report (including instrument setting and starting/sorted cell #s).

The flow lab will call when the sort is completed. It will take approximately 1 hr per 10 million cells.

RNA Extraction Prep

1. Collect the cells from the flow lab (still on ice).
2. Centrifuge (1200rpm, 7min, 4°C) to pellet the cells.
3. Label an RNase-free microcentrifuge tube with subject ID, cell population, and date.
4. Resuspend pellet in 700ul QIAzol Lysis Reagent.
5. Transfer to labeled microcentrifuge tube. Cap securely. Vortex for 1 min.
6. Store tubes at -70°C.

RNA Extraction

1. Remove NK cell (n=6) and CD8 cell (n=6) samples from -70°C freezer.
2. Allow samples to thaw and then incubate for 5 minutes at room temperature.
3. Add 140ul chloroform to each sample. Shake tube vigorously for 15 seconds. Incubate at room temp for 3 minutes.
4. Place each sample in the microcentrifuge in the cold room. Centrifuge at 12,000g for 15 minutes.
   There will be 3 phases: Upper, colorless, acqueous phase with the RNA
   Middle, white interphase
   Lower, red organic phase with protein
5. Collect 375ul of the upper, colorless phase and transfer to a new, labeled collection tube. Keep tip clear of the middle, white interphase and the lower, red organic phase. If there is extra volume in the upper phase you can collect it too, but make a note of the final volume. If there is not 375ul of volume in the upper phase, collect what you can without touching the white/red phases and make a note of the final volume. It is okay to estimate the final volumes if not 375ul.
6. Add 1.5 volumes (525ul) of 100% ethanol and mix thoroughly by pipetting up and down 6-7 times.
7. Pipet 700ul of sample onto a labeled, RNeasy Mini spin column.
8. Centrifuge spin columns at 9,000g for 15 seconds.
9. Remove columns, pour out flow-through and blot collection tube on paper towel. Re-insert column into collection tube and place on rack.
10. Open all column lids and add up to 700ul of remaining sample.
11. Centrifuge at 9,000g for 15 seconds. Discard flow-through as in step #10.
12. Repeat steps 11 and 12 as needed to pass all of the volume from each sample through the RNeasy mini spin column.
13. Add 700ul Buffer RWT to each spin column. Centrifuge at 9,000g for 15 seconds. Discard flow-through.
14. Add 500ul Buffer RPE to each spin column. Centrifuge at 9,000g for 15 seconds. Discard flow-through.
15. Add another 500ul Buffer RPE to each spin column. Centrifuge at 9,000g for 2 minutes. Transfer spin columns to new collection tubes and discard old tubes.
16. Centrifuge at 13,000g for 1 minute. Discard collection tubes.
17. Place spin column on labeled, 1.5ml tube. Carefully add 30ul of RNase-free water to the center of the column.
18. Incubate on benchtop for 1 minute.
19. Centrifuge at 9,000g for 1 minute.
20. Remove spin columns and discard. Close lids on collection tubes and place samples on ice.
21. Run Nanodrop on each sample. Use the Nucleic acid/RNA settings. Save to a new file.
    a. Run blank using the same bottle of RNase-free water.
    b. Run each sample twice using 1ul per test. If RNA concentration readings are off, run it a third time.
    c. Export a report as an Excel file to the L: drive under the following folder: _Rick/Documents/miRNA HIPC IOF. Label as “RNA Isolation YYYY-MM-DD” where YYYY-MM-DD is todays date.
22. If OD ratios look good: place samples in -80C freezer. If they look bad, run the RNA Cleanup procedure.

RNA Cleanup if needed

1. Adjust sample volume to 100ul with RNase-free water.
2. Add 350ul Buffer RLT and mix well.
3. Add 700ul 96-100% ethanol. Mix well by pipetting.
4. Transfer 700ul of sample to RNeasy MinElute spin column. Centrifuge at 9,000g for 15 seconds. Discard flow-through.
5. Repeat step #4 with remaining sample volume.
6. Add 700ul Buffer RWT to each spin column. Centrifuge at 9,000g for 15 seconds. Discard flow-through.
7. Add 500ul Buffer RPE to each spin column. Centrifuge at 9,000g for 15 seconds. Discard flow-through.

8. Add 500ul 80% ethanol to each spin column. Centrifuge at 9,000g for 2 minutes. Discard collection tube.
9. Place spin column on new collection tube. Centrifuge at 9,000g for 5 minutes. Discard collection tube.
10. Place spin column on labeled, 1.5ml tube. Add 14ul of RNase-free water to center of column. Let sit for 1 min.
11. Centrifuge at 9,000g for 1 minute. Discard spin columns. Close lids on collection tubes and place samples on ice.
12. Run samples on Nanodrop.
13. Place samples in -80C freezer.