

Lung MAP HTC - BioRepository for Investigation of Neonatal Diseases of the Lung (BRINDL)

Standard Operating Procedures

705.3_

Protocol: (Refer to 705.HTC_Staining_FACSort_LungCells) Micro-immunofluorescence Staining for Multichromatic FACS Sorting for major cell populations isolated from human lung tissue digests (refer to protocol 702.HTC_Lung_Tissue_Digestion)	
Worksheet 1.2: Immuno-fluorescence micro-method staining with multichromatic panel	
Date:	Operator(s):

Reagent/Materials/Equipment	Vendor/Catalog # or HIC reagent log	Lot # or page number	Expiration Date
PBS/1.0% BSA			
Panel Multi-stain cocktail			
Simply Cellular Anti-mouse compensation standard	Bangs Laboratory/ Cat#550		
Normal Mouse Serum	Sigma /M5905		
Stained Simply Cellular Anti-mouse compensation standard	Date Stained:	NA	2 days post stain
Sterile 5 ml Round-bottom FACS Tubes	BD Falcon/Cat# 352054		
DNase I from bovine pancreas	Sigma / Cat# DN25-5G		
Sterile 5 ml Round-bottom tubes with Cell Strainer	BD Falcon/Cat# 352235		NA
Rainbow Calibration Particles, Peak 6	Spherotech/RCP-30-5A-6		

Equipment/Model	Identifier
Centrifuge Model _____	
Pipettor (5-50µL)	
Pipettor (20-200µL)	
Pipettor (100-1000µL)	
Pipettor (1-10µL)	

Time	
Cells Thawed:	
Sort Began:	
Sort Ended:	
Samples frozen:	

Psort 1.0 Panel	Fluorophore	Dilution factor	Manufacturer/Cat#	Clone	Reagent volume used for today's sort (µl)
Podoplanin (T1α)	AF647	1:50	Biologend /337008	NC-08	
CD31 (PECAM1)	BV605	1:200	BD /562855	WM59	
CD326 (EpCAM)	PE	1:100	eBioscience /12-9326-42	1B7	
CD45	V450	1:50	BD /560367	HI30	
CD144 (VECadherin)	FITC	1:50	BD /560411	55-7H1	
CD235a	PE-Cy5	1:800	BD /559944	GA-R2	
7AAD	N/A	1:75	BD /559925		

_____ µl of reagents + _____ µl of staining buffer = _____ µl staining cocktail

Sample Preparation

1. Specimen samples are received protocol 702.HTC_Lung_Tissue_Digestion. Sample to be sorted could be fresh or from cryopreserved cellular preparations. If cryopreserved samples are used, follow protocol _____ for thawing and perform a cell count. Add information for cell samples used for the sorting procedure.

Specimen ID #	Sample Type fresh/frozen	Received From/ Time	Cell Count (cells/mL)	% Viability	Total Volume (µL)	Total # Cells/ tube

Immunofluorescence Staining of Cells and Controls:

1. Label eleven 5mL FACS tubes for the staining of a single sample (add one tube per each additional sample to be sorted). Add 5 µL of unstained cells from one sample and resuspend with 95 µl of PBS/1% BSA buffer. Set the tubes containing unstained cells aside on ice; it will serve as unstained cell control.
2. Add neat NMS to each **cell sample** to reach a final concentration of 2%. Incubate for 10 minutes at RT.

_____ µL of sample x 0.02 = _____ µL of NMS to add to tube

Sample ID	Volume of Sample (µL)	Volume of NMS (µL)

3. Remove **heat killed cells** from -80°C freezer and thaw in 37°C waterbath according to SOP _____. Place entire volume in 5mL FACS tube and place on ice. Add 1mL of staining buffer to tube.
4. Vortex Simply Cellular Anti-Mouse Compensation Standard bottle to resuspend and add 3 drops of **beads per compensation sample tube** except for the 7AAD tube, which contains the heat-killed cells. Add 1mL of staining buffer to tubes containing beads.

5. After the 10 minute incubation, spin all the tubes with the cells and beads in a sealed canister with inserts designed for 5mL round bottom FACS tubes for 6 minutes at 400X g at 4°C. Decant the fluid carefully into a waste container containing 10% hypochlorite solution in a biological safety cabinet, so as to not splash bleach into FACS tubes. Blot tubes on absorbent disposable toweling while inverted before returning to upright position, being careful not to disrupt pelleted cells/beads.
6. Gently tap tubes to resuspend pellet. In a dark biological safety cabinet, immediately add 50 µL of single stain diluted in PBS/1% BSA buffer containing 10 µg/ml DNase to the appropriate tubes with a fresh tip for each tube, mixing the cells or beads and solution in each well with the individual tip used to dispense the cocktail or buffer by pipetting up and down three times. Add the appropriate volume of Ab-Fluorochrome cocktail into sorting sample tube. The volume of Ab-Fluorochrome cocktail is recorded below and is based on the ratio of 1x10⁶ cells/10 µl Ab-Fluorochrome cocktail.

Sample ID	Cocktail Volume (µL)

7. Incubate tubes for 80 minutes at 4°C in the dark.
8. Pre-coat collection tubes with 3 ml of PBS/1% BSA and 10 µg/ml DNase buffer using sterile conditions. stained for at least 30 minute at room temperature. Invert tubes several times during the incubation period to ensure complete coating.
 - a. If using the one-step sort protocol (for isolating 4 cell populations), then coat one tube per each million cells stained.
 - b. If using the two-step sort protocol (for isolating 7 cell populations), then coat one tube per 750,000 cells stained.
9. After the 80 minute incubation, add 2 mL PBS/1% BSA and 10 µg/ml DNase buffer at 4°C to each tube from step 7. Spin tubes for 6 minutes at 400X g at 4°C. Decant supernatant into a waste container and blot the last drop on absorbent toweling.
10. Tap tubes from step 9 to resuspended cells and beads. Re-suspend unstained beads, unstained cells, stained beads, and 7AAD compensation controls with 200 µL PBS/1% BSA and 10 µg/ml DNase buffer. Resuspend stained sorting sample with 200 µL HBSS/1% BSA buffer. Flick tubes to re-suspend cells and beads.
11. Pre-wet 5 mL round-bottom filter tubes for the **cell samples** and 7AAD control by adding 500 µL HBSS/1% BSA buffer to tubes making sure the entire filter gets rinsed by swirling the pipet tip to wet the entire surface of the filter. Remove the liquid in the tube by decanting. Sterilely

transfer stained **cell sample** and 7AAD compensation control each to a 5 ml tube with a pre-wetted cell strainer cap to filter out clumps. Add 100 µL HBSS/1% BSA buffer using a fresh sterile tip to the original tube to rinse and add then add to tube containing cell strainer to rinse strainer cap. (Do not exceed a concentration of 50×10^6 cells/ml. Dilute in a higher volume if necessary.) After rinsing, replace the cell strainer cap with the white cap from the original staining tube so that it is sealed and can be safely vortexed on the bench in the sorting facility.

12. Add 200 µL HBSS/1% BSA buffer to a round-bottom FACS tube for the addition of Peak 6 beads once in FlowCore.
13. Bring all the samples on ice to the flow core in a sealed container.
14. Vortex the Peak 6 beads vigorously and add 1 drop to the tube prepared in step 12. Record the lot number of the Peak 6 beads on page 1 of this worksheet.

Running the Sorter (Instructions for the Sorter Operator):

1. Verify that the 85 µm size nozzle and temperature setting of 4°C are used. Currently, only Waldorf can be used to perform sorts.
2. Open a new experiment in FACSDiva by selecting “new experiment” from the dropdown menu. The following message will pop up “The settings from CST are different from those on the cytometer. Do you want to use the CST values?” Select “Use CST settings”.
3. Right click on global worksheet in the browser and select “new global worksheet”. In the browser window, right click on global worksheet 1 and select “apply analysis template”. Select the appropriate sort template under the general tab.
 - a. If using the one-step sort process, then use the template LungMAP030416 and application setting LungMAP031416.
 - b. If using the two-step sort process, then use the template LungMAP 9_3_13. The first sort step will be used to isolate cells of immune, lymphatic, Type I epithelial, and mixed origin. The mixed cells origin cells are then spun down, resuspended in 250 µl and rerun using the second global worksheet to isolate the remaining epithelial, endothelial, and mesenchymal cells.
4. Apply application specific settings. Record the specific template name, Sorter, and application specific setting below.

Template Name	
Application Specific Setting Name	
Sorter	

5. Select global worksheet 2. Vortex the tube containing the Peak 6 beads and then run the Peak 6 beads prior to any other controls or specimen samples to confirm instrument voltage settings yield target fluorescent values. Collect 5,000 events within the P1 gate. The MFI (mode fluorescence intensity) of the specific lot of peak 6 beads in each channel should not exceed the

acceptable range established for the specific sorter. **NEED TO PUT IN SOMETHING ABOUT TRACKING MODE VALUES FOR TRACKING INSTRUMENT AND RE-BASELINING INSTRUMENT IF NECESSARY (either due to new lot of peak 6 or maintenance was performed).**

6. Select the global worksheet 1 and then select unstained cells. Vortex unstained cell control with cap on, and then run the unstained cell control followed by the fully stained sample after vortexing. Collect and record 10,000 total events each to verify both the positive and negative populations in each channel are on scale with the application specific voltage settings used. 95% of the cells should be below 10^5 in each fluorescent channel.
 - a. NOTE: Forward and side scatter plots should be set to log scale. Furthermore, as some epithelial cells are extremely small, care must be taken to include these cells in the sort by recording a very low threshold value (e.g. 5,000).
7. Run single stain controls on the linear scale. Make sure each sample is vortexed before running. For unstained beads, 7AAD control and single stained beads, collect 10,000 (minimum) to 15,000 (optimal) for the single stained beads and 50,000 events for the 7AAD control stained cells.
8. Select the normal worksheet and verify the placement of the P2 gate on the compensation controls. First adjust the P1 gate on the unstained control tab to include the majority of beads for the compensation controls. Apply the P1 gate to all bead controls by right clicking on the P1 gate and selecting “apply to all compensation controls”. The P1 gate will need to be moved manually for the 7AAD control since these are cells and not beads. Then verify the place of the P2 gate for each compensation control but selecting each the specific tab for each control and verify the positive population is included in the P2 gate. The gates can be moved manually if the entire population is not contained within the gate. Calculate the compensation matrix by selecting experiment in from the drop-down menu and select compensation setup. Select “calculate compensation” and another window will pop up. Rename the compensation matrix the same name as the experiment and select “link and save”. Return to the global worksheet
9. Select the sample in the browser window. Collect 20,000 events of the fully stained sample. The investigator will confirm the gating is compliant with the pre-determined gating scheme. Note that the size of gates will be large in order to maximize cell recovery. Turn off bi-exponential transformation once the gating is verified.
10. Open sort layout, select 4-tube and mask 4-way purity. Set the number of cells being collected per tube at 1×10^6 for each of the following populations. Verify the collection tubes are positioned (far left, left, right, far right) based on their frequencies from the test run result so that the two most abundant populations are in the left or right positions whereas the two least abundant populations are in the far left or far right positions to minimize contamination.
11. Place the collection tubes in the sorter and set the events to be recorded for the sorting layout FCS file at 2,000,000 in the acquisition dashboard. Start sorting the first specimen tube. Select acquire in the acquisition dashboard and verify events are being collected and collection appears

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normal based on the gating scheme. Select record. Once any of the cell populations reach 1×10^6 cells, stop the sort. Replace the appropriate collection tube with a labeled pre-coated new collection tube, modify the cell numbers established to reflect what has already been collected for the other populations so total number of cells for the other populations being collected does not exceed 1×10^6 and continue the sort. Cap and place tubes containing sorted cells on ice.

12. Prepare the PDF of sort report and layout (gating scheme). Transfer the PDF along with the FCS files into the appropriate folder (e.g. the Pryhuber lab folder on the transfer pipeline).
13. Call the investigator(s) to retrieve the sample(s) once all the samples are completed.
14. The investigator will process the cells using the appropriate SOP for downstream analyses (e.g. lyse, freeze or cytopins resulting cell populations)
15. The investigator should transfer the folder that contains all of the electronic files (fsc, sort layout, and sort reports) for the day's sort into the LungMAP data storage folder.

Sorted on Date: _____ **Operator:** _____

File name _____ **Archive location:** _____

Post Sort Cell Yields:

Cell Type	Yield	Post-sort Cell Treatment

Daughter Vial Labels	Cell number/Daughter Vial	Additive

Comments: _____

Revision History

Version	Changed By	Purpose	Change Date:
705.3	Gautam Bandyopadhyay	Added tables to put Post-sort cell yields and daughter vial description, a time record on Page one, additional reagent volume information on page 1. Updated antibody catalog numbers (1 st page) and Instrument template and application setting.	07/26/17
705.2	Gautam Bandyopadhyay	Addition of DNase in sorting buffer	05/08/17
6XX.01	R Misra	Created based on the URM Human Immunology Core SOP# 1-0054 for isolating FACs purified PBMC populations	01/11/15