University of Pittsburgh Cancer Institute
Flow Cytometry Facility
Hillman Cancer Center Research Pavilion

5117 Centre Ave
Rooms 1.45 & 1.47
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Procedure Name: FCL 03-01-R0 PROCEDURE FOR EXTRACELLULAR STAINING

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Date Revised: NA **Revision number:** 0

Authors: Singer CL, Donnenberg VS, Donnenberg AD

Supersedes Procedure: NA

Distribution: Flow Cytometry Laboratory, Flow Cytometry Users

Laboratory Director_____**Date**_____

Associate Director_____**Date**_____

Technologist Annual Review

Date	Signature

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03-XX-R0 PROCEDURE FOR

Principle

Specimen or Component Requirement

Reagents and Supplies

Supplies	Supplier	Catalog number
Ca++ Mg++-free phosphate buffered saline	Gibco	70013-32
Other ingredients for media		
96-well round bottom microtiter plates	Rainin	
12 x 75mm snap cap tubes		BD
Methanol free formaldehyde		Polysciences

Instrumentation

Item	Manufacturer	Model number
Centrifuge		
96-well plate carriers		

Procedure

Cautionary Notes

When flicking the plate after centrifugation (step 3), make a single smooth motion. Hesitating, or flicking twice will result in loss of cells.

Tandem dyes (e.g. ECD, PC5, PC7) are very sensitive to ambient light. Not only are they susceptible to photobleaching, but they can also break down, resulting in free

PE. This can wreak havoc with compensation. Make an effort to protect your antibodies and stained cells from light as much as possible.

The Beckman-Coulter XL Cytometer is very fussy about tubes. 12 x 75 mm tubes (polystyrene [clear] or polypropylene [translucent]) manufactured by BD or Beckman Coulter fit well. Other tubes (e.g. Fisher brand), seem to fit, but cause excess wear on the sample head (an $800 part). Please use only BD or Beckman-Coulter tubes.

Bulk staining: When two or more antibodies are shared by a large number of tubes in a panel, it is cost-effective to stain the cells in two steps. For example to stain a 10-tube panel in which all tubes share CD4 and CD8, first stain 10 million cells in a single well using 1-2 μL of each antibody. Then divide the cells into 10 wells and stain each well with the remaining unique antibodies. Bulk staining can also be used effectively when large numbers of cells are stained for cell sorting or rare event detection. Remember: the antibody concentration in the reaction mixture is all that counts. Target molecules on cells are outnumbered by antibody molecules by many orders of magnitude. Even the total number of cells is insignificant, up to about 10 million cells/well.

Detailed Methods

Staining Cells

1. Resuspend cells in 1ml of staining medium at a concentration of 2-50 x 10^6 cells/mL. The concentration depends on the number of cells to be deposited per well in a volume of 100 μL. We like to stain at least 200,000 cells per well. This protocol works well for up to 5 million cells/well.
2. Pipette 100 μl of cell suspension per well into 96 well polystyrene round-bottom, plates. Centrifuge plate for 10 min at 1600 RPM (400g) at room temperature.
3. Decant supernatant by gently flicking plate over sink and blotting on a paper towel. This will leave on average 10-15μl residual volume/well.
4. Add antibodies to well. Most preparations work well at 1-2 μl/well. One μl of antibody in this protocol is equivalent to 10 μl in manufacturer's recommended staining procedures in which cells are resuspended in 100 μl. **The critical factor is the antibody concentration, not the number of cells/well or the absolute amount of antibody/well.** Total volume must be kept below 20μl/well.
5. Cover plate with aluminum foil. Incubate 30 min at 4°C.
6. Wash 1X: Add 200μl/well of staining medium and centrifuge plate for 10 min at 700 RPM. Decant supernatant as in step 3.
7. Transfer to 12x75mm snap cap tubes and adjust the volume to approximately 3ml of PBS, and wash again by centrifugation.
8. Deacant. Add 1.0 mL of formaldehyde solution.
9. Store away from light at 4°C until sample is acquired on the cytometer.

Adapted from procedures, complements of Donnenberg Lab, University of Pittsburgh

Result Reporting

Not applicable

Quality Control

References

This procedure developed by the Flow Cytometry Laboratory.

Appendices

See following examples.

Revision	Change	Rationale	CFR/FACT Standards	Start Date	End Date
0	Creation	New procedure.	D5.100		
1					
2					
3					
4					