UNIVERSITY OF ROCHESTER MEDICAL CENTER

Human Immunology Center Core Laboratory
David H. Smith Center for Vaccine Biology and Immunology
Aab Institute of Biomedical Sciences

STANDARD OPERATING PROCEDURE: Immunofluorescence Staining for sorting of specific cell populations with Human Single Cell Suspensions.

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Purpose and Scope:

This procedure describes a method for multichromatic staining of human single cell suspensions with up to seven antibody fluorochrome conjugates for sorting using the BD FACSAria fluorescent antibody cell sorter located in the CVBI consortium or up to 12 colors in the CPBR Flow Laboratory. This method is used by the Human Immunology Core Laboratory in exploratory clinical research studies for sorting specific human cell populations based on cell surface characterization.

Principle:

Single cell or peripheral blood mononuclear cells are stained with cell surface reactive antibodies conjugated to fluorochrome. Antibody fluorochrome conjugates with up to seven different fluorescent light emissions at different wavelengths may be used to detect cell surface markers simultaneously on human cells with the BD FACSAria cell sorter. Single cell suspensions or peripheral blood mononuclear cells are prepared from human specimens and stained with either direct antibody (Ab) fluorochrome conjugates or antibody-biotin conjugates followed by strepavidin-fluorochrome conjugates . Control cell suspensions or antibody capture beads are individually stained with a single Ab fluorochrome conjugate of each antibody fluorochrome used in the multiple stain reactions to compensate for the spill over in the emission spectrums for each fluorochrome. This allows the instrument to calculate and subtract the appropriate overlap to yield the specific signal intensity for each fluorochrome. After sorting the cells are resuspended in media and used for further clinical analysis.

References:

Current Protocols in Immunology (2001) 5.3.1-5.3.24 John Wiley & Sons, Inc.
Peter Schenkle et al. Evaluation of a Novel Mononuclear Cell Isolation Procedure for Serological HLA Typing. Clin. Diag. Lab Imm. Nov. 1998 p.808-813.
Current protocols in Immunology (1996) 7.1.1-7.1.3 John Wiley & Sons, Inc.
Recommendations for Prevention of HIV Transmission in Health-Care Settings: Universal Blood and Body Fluid Precautions Guideline CDC 1987
CDC Biosafety in Microbiological and Biomedical Laboratories 4th edition U.S.H&HS, Public Heath Service
HIC-1-0007 current revision: Cryopreservation of cells: Freezing and thawing
HIC-1-0019 current revision: Isolation of Human Peripheral Blood Mononuclear Cells using BD CPT tube

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HIC-1-0020 current revision: Isolation of Human Peripheral Blood Mononuclear Cells using Ficoll-Hypaque Density Gradient Centrifugation
Current Protocols in Immunology (2007) 3.6.1-3.6.2.0 John Wiley & Sons, Inc.

Safety:

Personnel will adhere to safe work processes outlined in U.S. Public Health Universal Precautions Guidelines for use of human blood and body fluids and follow biosafety level 2 (BSL2) practices.

Reagents and Material:

FACSFlow Sheath Fluid buffer Cat. # 340398 or equivalent
FBS Cellgro Cat. # 10-040-CV or equivalent
Staining buffer: RPMI/8%FBS (R8)
Fluorochrome-labeled, biotin-labeled antibodies and fluorochrome –labeled streptavidin for flow cytometry applications
BD Allegra X-12 centrifuge with SX4750 swinging bucket rotor and inserts with seals or equivalent
Hemocytometer or automated cell counter
0.4% Trypan blue exclusion dye in saline
Biological Safety Cabinet, Class II
Household bleach (5% sodium hypochlorite)
Absorbent towels
Sterile serological pipets 10, 5, 1 mL size
Pipet aide for serological pipets
Sterile pipet tips
Digital variable single channel air displacement pipets 5-50 μL, and 30-300 μL
BD CPT tubes Cat. # 362753 or standard blood tubes with heparin for blood collection and processing
Waste pan
10% bleach solution
variable speed vortex mixer
sterile 15 mL & 50 mL conical polypropylene tube
Ice bath for tubes
Test tube racks for 12 X 75 mm tubes, 15 mL and 50 mL conical tubes
12 X 75 mm 5 mL plastic tubes with closures, VWR International Cat # 60818-500 or equivalent
Ficoll-Hypaque solution for peripheral blood mononuclear cell purification (or BD CPT tubes will serve for collection an PBMC purification)
Simply Cellular Compensation Beads, Bang Catalog # 550 or appropriate human cells for compensation controls.
5mL tube with cell-strainer cap, BD Falcon Cat.# 352235, or equivalent

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Reagent Preparation:

Prepare Staining buffer (RPMI): Remove 40 mL of RPMI from 500mL bottle and replace with 40 mL of FBS. Recap and invert bottle gently 3 times to mix avoiding bubble formation. Record preparation date and preparer initials on container and all preparation information in a notebook or reagent logbook.

Procedure for Blood Processing:

* All work is performed using BSL 2 procedures and following universal precautions for handling human blood and body fluids.

Blood is collected by venipuncture either using BD CPT tubes or in BD vacutainer tubes with heparin (8 ml capacity, heparinized is preferred). After collection, blood must be separated by Ficoll hypaque density gradient within 8 hours or CPT centrifugation within 2 hours. The mononuclear cell layer of the specimen are harvested and used immediately for staining and sorting. Process blood using either SOP HIC-1-0019 for CPT or SOP HIC-1-0020 for Ficoll-Hypaque density gradient separation.

For using frozen cells:

1. Follow procedure described in SOP HIC-1-0007.2

Immunofluorescence Staining of cells for Multichromatic FACS analysis

*If frozen cells are used for staining controls or compensation, a standard thaw procedure must be employed to insure cell viability. Reference HIC-1-0007.2

The peripheral blood mononuclear cells or single cell suspensions are stained for sorting and used immediately for sorting on the BD FACSAria. The cell number is typically 80 -100 $x10^6$ cells for sample sorting. Either cells (1 $x10^6$) or antibody capture beads may be used for single stained compensation samples. The following procedure is used to stain the cells. For tandem dyes the same conjugate lot used in the multichromatic panel should be used for the compensation sample.

1. Label (12 X 75 mm) round bottom tubes with the test sample identifier and the appropriate experimental conditions: unstained cells and the appropriate single stained antibody/fluorochrome conjugate (one for each compensation control). Compensation controls may be run with either the control cells or with antibody capture beads.
2. Perform all steps from this point forward in a dark biological safety cabinet.
3. Add R8 staining buffer to each tube to be stained to qs to the final volume for the panel (generally in the range of 50 to 100 µL for the compensation controls and unstained control and 1mL for the sample to be sorted) or R8 only to the unstained control well/tube. Using the appropriate size pipet for the volume to be dispensed and a fresh sterile tip, add the previously

determined optimal amount of single Ab fluorochrome conjugate or multichromatic panel to the respective microtiter well/tube. The concentration used is in most cases the same as the maufacturers recommended final concentration with a final volume of 1 mL the 80 to 100 million cells

4. Gently mix the tube containing the PBMC or single cell suspension on the low speed of the vortex mixer. Using a new sterile tip for each tube or well, add the appropriate amount of cell suspension to each of the labeled tubes. Gently mix the tubes by tapping the tube with fingertip.
5. Incubate the tubes in the dark on ice for 20 minutes unless otherwise specified in an individual test protocol.
6. Wash by adding 1 mL R8 at 4^0C to each tube. Centrifuging the tubes for 6 minutes at 4^0C at 300 X g (1100 RPM). Decant supernatant into a beaker containing 10% bleach solution and gauze (never decant directly into fluid). Repeat wash one time
7. If using a biotin/strepavidin system, add 100 μL of streptavidin-fluorochrome conjugate in R8 at the optimal dilution to the single stained tube and add 1 mL of streptavidin-fluorochrome conjugate in R8 at the optimal dilution to the sort sample tube. Place on ice and incubate in the dark for 20 minutes. For tubes without biotin conjugate, proceed directly to step 9.
8. Wash the tubes twice as in step 5 to remove excess streptavidin-fluorochrome conjugate
9. Resuspend in 200 μL of R8 for the unstained control and the compensation controls. The sample should be resuspended in 1mL of R8. Samples are sorted on the BD FACSAria immediately.
10. Place 1mL of media in the desired number of tubes for collection on the FACSAria. Sorting should be performed under BSL-2 guidelines and sterile conditions.
11. Sample should be filtered prior to sort to prevent clogging of the FACSAria.

Revision History

Version	Change	Impact	Justification	Change Date:
HIC-1-0021	new			09-06-07