UNIVERSITY OF ROCHESTER MEDICAL CENTER

Human Immunology Center Core Laboratory
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STANDARD OPERATING PROCEDURE NUMBER HIC-1-0004

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Title:
Micro-immunofluorescence Staining for Multichromatic FACS Analysis with Human Single Cell Suspensions

Purpose and Scope:

This procedure describes a micro-method for multichromatic staining of human single cell suspensions with up to eleven antibody fluorochrome conjugates for analysis using the BD LSR II fluorescent antibody cell sorter. This method is used by the Human Immunology Core Laboratory in exploratory clinical research studies to analyze human clinical specimens for immune status and cell surface characterization.

Principle:

Single cells or peripheral blood mononuclear cells are stained with cell surface reactive antibodies conjugated to fluorochrome in a v-bottom microtiter plate with the cell pellet and 10 to 15 μL of Ab-fluorchrome(s) solution. Antibody fluorochrome conjugates with up to eleven different fluorescent light emissions at different wavelengths may be used to detect cell surface markers simultaneously on human cells with the BD LSR II flow cytometer. 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 or Molecular Probes, Zenon technology kits. 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 staining the cells are resuspended in isotonic solution containing 2% formaldehyde to fix and transferred to flow cytometer compatible tubes for FACS analysis. The cells may be analyzed by flow cytometry up to 48 hours following staining.

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

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
Bovine serum albumin (BSA) Fraction V ICN Cat. # 160069 or equivalent
Staining buffer: 1X Hank's balanced salt solution (HBSS) with 1% W/V BSA
Fluorochrome-labeled, biotin-labeled or Zenon technology labeled antibodies and fluorochrome – Fluorochrome-labeled streptavidin for flow cytometry applications
BD Allegra X-12 centrifuge with SX4750 swinging bucket rotor and inserts with seals
Hemocytometer or automated cell counter
0.4% Trypan blue exclusion dye in saline
Fixation Buffer: FACSFlow with 2% formaldehyde
37 % Formaldehyde, Sigma Cat. # F-1635 or equivalent
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
Waste pan
10% bleach solution
variable speed vortex mixer
sterile 15 mL & 50 mL conical polypropylene tube
v-bottom polystyrene microtiter plates (Nunc Cat # 249662 or equivalent)
Ice bath for tubes or microtiter plates
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
Simply Cellular Compensation Beads, Bang Catalog # 550 or human cells for compensation controls.

Reagent Preparation: Record preparation date and preparer initials on container and all preparation information in a notebook or reagent logbook for each reagent.

Staining Buffer: Prepare Staining buffer (sterile HBSS/1% BSA): Measure 75 mL BD FACSFlow BD and add 750 mg BSA in a sterile plastic container. Gently mix by inverting with cap in place until protein is dissolved. (1% = 10 mg/mL). Sterile filter the buffer using a 0.2 μ filter/sterile pyrogen free plastic flask and store at 4^0 C. Handle aseptically.

Fixation buffer: Prepare (FACSFlow/2% para-formaldehyde): 49 mL FACSFlow with 1 mL formaldehyde (37%).

Blocking Buffer: Prepare 5% normal mouse serum in sterile staining buffer by adding 50 μL of normal mouse serum to 1.0 mL of sterile staining buffer. Reagent may be keep at 4^0 C for up to one month.

Streptavidin fluorochrome-conjugates: Store SA-conjugates in single use aliquots at -80^0 C. Immediately before use dilute to the optimal dilution in staining buffer in the dark.

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 centrifuged or separated by density gradient centrifugation within 2 hours using either ficoll hypaque or CPT tube method. The mononuclear cell layer of the specimen may be harvested and used within 48 hours of collection if stored at 4^0 C in sterile HBSS/1% BSA or within 24 hours if re-suspended in plasma in CPT tube and stored at 4^0 C.

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.

The peripheral blood mononuclear cells or single cell suspensions are blocked with 5% normal mouse serum before staining with antibody-fluorochrome. The fixed stained cells may be stored up to 48 hours at 4^0 C before analysis on the BD LSR II FACS. The cell number per v-bottom microtiter well should be between 1.0×10^5 to 1×10^6 cells. A positive control specimen (normal human donor) is stained with each set of unknown specimens to assure method and reagent performance. Either cells or antibody capture beads depending on the species specificity of the antibody 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. If more than one antibody panel is run simultaneously, multiple compensation samples may be required with tandem dyes for adequate compensation.

1. Prepare a template for the microtiter plate and label with the control or test sample identifier and the appropriate experimental conditions: unstained cells, appropriate single stained antibody/fluorochrome conjugate (one for each compensation control), or each Ab/fluorochrome cocktail panel to be tested. Compensation controls may be run with either the control cells or with antibody capture beads. When using beads, shake bottle well to resuspend and use one drop of beads per well (@ 50 μL). Stained beads may be used for compensation purposes for 48 hours as long as they are kept at 4 ^{0} C in the dark and are used with panels made from the same lot of Ab-fluorochrome.

2. Gently mix the tube containing the PBMC or single cell suspension on the low speed of the vortex mixer or by hand. Pipet 50 to 150 μL of test or control cells into the appropriate V-bottom wells of the microtiter plate using a fresh tip for each cell specimen. Add 50 μL of 5 % normal mouse serum in staining buffer to each cell specimen. Incubate for 10 minutes at room temperature. Spin the plate in a sealed microtiter cup holder for 6 minutes at 400 X g. Decant the fluid into a waste container in a biological safety cabinet and blot the plate on absorbent disposable toweling or aspirate with an 8-channel microtiter aspirator with sterile fresh tips.

3. Perform all steps from this point forward in a dark biological safety cabinet.

4. Immediately add the designated volume (10 to 15 μL) antibody-fluorochrome conjugate cocktail, single stain or HBSS/1% BSA staining buffer to the appropriate wells with a fresh sterile tip for each well, mixing the cells and solution in each well with the individual sterile tip used to dispense the cocktail or buffer by pipeting up and down three times.

5. Incubate the tubes or plate in the dark on ice for 20 minutes unless otherwise specified in an individual test protocol. Certain panels, such as the T cell chemokines, may require a 20 minute incubation at 37^0 C in the dark.

6. Wash by adding 200 μL staining buffer at 4^0 C to each well. Centrifuging the plate for 6 minutes at 4^0 C at 400 X g. Decant supernatant into a waste container containing 10% bleach solution and gauze (never decant directly into fluid) followed by blotting the last drop on absorbent toweling before inverting upright. Repeat wash one time. Alternately, aspirate the wells of the microtiter plate using an 8 channel aspirator with clean tips.

7. If using a biotin/strepavidin system, add 10 μL of streptavidin-fluorochrome conjugate in staining buffer at the optimal dilution to the single stained tube/well and to the multichromatic cocktail tubes/wells. Place on ice and incubate in the dark for 20 minutes. For tubes without biotin conjugate, proceed directly to step 9.

8. Wash the microtiter wells twice as in step 6 to remove excess streptavidin-fluorochrome conjugate.

9. Resuspend in 200 µL of fixation buffer (FACSFlow with 2% formaldehyde) and leave in microtiter plate for reading using the High Throughput Autosampler System on the LSRII or transferred to either large volume microwell tubes or to 12 X 75 mm tubes for manual reading. Samples may be analyzed on the BD LSR II FACS immediately after fixing or stored at 4^0C for up to 24 hours if 7-AAD live/dead stain is used or up to 72 hours depending upon the fluorochrome used before reading. If using 12 X75 mm tubes, add additional FACSFlow buffer to make @ 0.5 mL final volume immediately before reading on LSR II. Samples from microtiter wells may be transferred to microwell tubes that will fit into a 12 X 75 mm size tube for reading on the LSR II.

Data Analysis and Interpretation:

Data is collected and auto-compensated using FACS Diva software on the LSR II. The FCS files are transferred onto the HIC server into individual accounts and a back up data disc is burned from the BD LSR II computer D drive. Files are analyzed using Flowjo software program.

Revision History

Version	Change	Impact	Justification	Change Date:
HIC-1-1004draft				12-29-2005
HIC-1-1004	new		Finalized SOP and added Normal mouse blocking step; HTS reading option; improved instructions for preparing reagents.	12-27-2006