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STANDARD OPERATING PROCEDURE: Immunofluorescence Staining for Multichromatic FACS Analysis 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 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 cell or peripheral blood mononuclear cells are stained with cell surface reactive antibodies conjugated to fluorochrome. 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. 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

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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 – labeled streptavidin for flow cytometry applications
BD Allegra X-12 centrifuge with SX4750 swinging bucket rotor and inserts with seals
Hemocytometer
0.4% Trypan blue exclusion dye in saline
Fixing 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
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
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
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 human cells for compensation controls.

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Reagent Preparation:

Prepare Staining buffer (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). Make fresh daily or prepare a sterile stock using 0.2 micron filter and store at 4 degrees C . Prepare Fixing buffer (FACSFlow/2% formaldehyde): 49 mL FACSFlow with 1 mL formaldehyde (37%). 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 centrifuged or separated by Ficoll hypaque density gradient centrifugation within 2 hours. The mononuclear cell layer of the specimen may be harvested and used within 48 hours of collection if stored at 4 degrees C in sterile HBSS/1% BSA or within 24 hours if re-suspended in plasma in CPT tube and stored at 4 degrees C.

For CPT tubes:

1. Gently invert the BD CPT tube containing 8 mLs of blood 8 to 10 times to mix before centrifuging using the tube inserts (must be cushioned to prevent breakage) for 16 X 125 mm tubes in sealed cups for 30 minutes at 2500 to 2800 rpm (1500 X g) at 20^0 C. An equivalent weight balance tube is used as a counterbalance.
2. The separated blood is removed from the sealed centrifuge cup inside a biological safety cabinet. The cap is removed and placed in a disinfectant waste pan containing 10% bleach solution. Using a sterile 5 mL serological pipet, remove 5 mL of plasma and platelets above the buffy coat layer and discard into waste pan.
3. With a fresh sterile 5 mL serological pipet remove the buffy coat into a sterile 15 mL conical tube. Wash tube and gel surface with 2 mL HBSS buffer and add to cells in 15 ml tube. Wash the cells by adding HBSS buffer to 15 mLs and centrifuge for 10 minutes at 1100 RPM (300 X g) at 4^0 C.
4. Remove the supernatant with a 10 mL sterile serological pipet being careful not to disturb the cell pellet and discard in the waste container. Repeat the wash in step 3 two times to remove most platelets.
5. Resuspend cells by adding the proportion of staining buffer (HBSS with 1% BSA) of 1 mL buffer per 8 ml blood volume to the pellet. Remove 10 µLs with a sterile tip and mix with 90 µLs of 0.4% trypan blue exclusion dye. Count viable and non-viable cells and record cell number.

6. Adjust the concentration to yield no more than 2 X 10^7 cells per ml if necessary and then add 10 µLs of normal mouse serum as Fc receptor block for every 1.0 mL of cell suspension. Incubate cells with mouse serum for 10 minutes at room temperature. The cell suspension is now ready to pipet into microtiter wells or test tubes. A 50 µL aliquot should contain approximately 1 X 10^6 cells (the minimum number of cells per well or tube for staining is 300,000). Specific test protocols will specify the number of cells required to be stained per panel.

For Ficoll-Hypaque separation:

1. Dilute whole blood with an equal volume of 1X Dulbecco's phosphate buffered saline in a sterile container. Layer diluted blood 10 mLs on to 3 mLs of Ficoll-Hypaque in a 15 mL tube or 35 mL on to 17.5 mL of Ficoll-Hypaque solution by dribbling down the side of the tube using a 10 mL pipet with a pipetman on slow speed.
2. Balance tubes and place in centrifuge cups with lids to seal. Centrifuge at 900 X g (2500 RPM in the Allegra) for 30 minutes at 18 to 20 degrees C with no brake.
3. After centrifuge has stopped, remove tubes from the centrifuge cups in the hood being careful not to disturb the white cell layer on top of the ficoll layer. Remove the white cells by siphoning with a 5 mL pipet and place in a 15 or 50 mL tube. Centrifuge the harvested cells at 300 X g (1100 RPM) for 10 minutes at 4^0 C. Pour off supernatant into waste and tap up cells in pellet. Wash 2 times by resuspending cells in 10 to 20 mL HBSS and centrifuging at 300 X g (1100 RPM) for 6 minutes at 4 ^{0} C to remove platelets.
4. Continue as in step 5 and 6 above for CPT processing.

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 stained for immunophenotyping and 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 staining tube should be between 2.5 X 10^5 to 1 X 10^6 cells per well or tube. 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 may be used for single stained compensation samples. The following procedure is used to stain the cells (decrease number of wells or tubes for compensation appropriately if fewer than 11 colors are used). For tandem dyes the same conjugate lot used in the multichromatic panel should be used for the compensation sample. This may require multiple compensation samples for different panels being run;

1. Label (12 X 75 mm) round bottom tubes or a 96 well template with the control sample 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.

2. Perform all steps from this point forward in a dark biological safety cabinet.
3. Add HBSS/1% BSA staining buffer to each well/tube to be stained to qs to the final volume for the panel (generally in the range of 50 to 100 μL) or buffer 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.
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 50 μLs of the cell suspension to each of the labeled tubes or wells of a v-bottom microtiter plate. Gently mix the tubes by tapping the tube with finger tip. Mix each well in the microtiter plate with the individual sterile tip used to dispense the cells.
5. Incubate the tubes or plate in the dark on ice for 20 minutes unless otherwise specified in an individual test protocol.
6. Wash by adding 1 mL staining buffer at 4^0C to each tube or 100 μL of staining buffer to each well in the microtiter plate. Centrifuging the tubes or plate 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) 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 100 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 tubes or microtiter wells twice as in step 5 to remove excess streptavidin-fluorochrome conjugate.
9. Resuspend in 200 μL of fixation buffer (FACSFlow with 2% formaldehyde). Samples may be analyzed on the BD LSR II FACS or stored at 4^0C for up to ????72 hours. 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 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

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Version	Change	Impact	Justification	Change Date:
HIC-1-1001.1	Typographical errors were corrected	none		07-08-05
HIC-1-1001.2	Staining buffer was changed from FACS flow to HBSS without phenol red	Optimum cell viability will be maintained	This switch was made through out the Mosmann laboratories. This is not a critical change.	07-18-05
HIC-1-1001.3	1. Addition Normal mouse serum to block non-specific Fc receptors binding of fluorochromes to the cells. 2. v-bottom microtiter plates were added as an option to staining in tube format. 3. upgrade from 8 to 11 color multichromatic configuration	Will prevent or minimize nonspecific binding of Ab fluorchrome conjugates through the Fc receptor binding onto monocytes and other cells Use of V-bottom plates allows the staining of increased number of samples.	Kelli Fudella HIC Research Notebook Number-1	11-16-05