Becton Dickinson Procedures

Direct Immunofluorescence Staining of Cell Surfaces in Whole Blood

This method is used to detect cells bearing specific membrane antigens by treating whole blood with monoclonal antibodies conjugated to fluorescein (FITC) or phycoerythrin * (PE). Direct staining is recommended for analysis by the Fluorescence Activated Cell Sorter (FACS™) or FACS Analyzer.

Reagents:

1. FITC or PE conjugated monoclonal antibody to human membrane antigen.
2. Lysing Solution

   10X Stock Solution:

   Dissolve in 1 liter of glass distilled water

   82.9 g NH_4Cl
   10.0 g $KHCO_3$
   370.0 mg monosodium EDTA

   Adjust to pH 7.3. Store at 2° to 8°C in a tightly closed bottle.

   1X Working Solution:

   To 90 ml glass distilled water, add 10 ml 10X Lysing Stock Solution. Mix well. Store at room temperature. Discard after one week.
3. Hank's Balanced Salt Solution containing no calcium or magnesium (HBSS), or Phosphate Buffered Saline (PBS) Solution.

   25X Stock Solution:

   Dissolve in 1 liter of glass distilled water

   188.0 g K_2HPO_4
   33.0 g $NaH_2PO_4 \cdot H_2O$
   180.0 g NaCl

   Store at room temperature.

   1X Working solution:

   To 960 ml glass distilled water, add 40 ml of 25X Stock Solution. Add 1.0 g sodium azide. Mix well. The pH should be 7.2 ± 0.2. Store at 2° to 8°C. Use cold buffer for diluting and washing cells.
4. 1% Paraformaldehyde

   Dissolve 1 g paraformaldehyde (Baker S-898-7) in 100 ml of appropriate diluent recommended for the flow cytometer or in 1X PBS.

Equipment and Materials:

1. EDTA Vacutainer™ tubes (Becton-Dickinson)
2. 12 x 75mm test tubes (Falcon 2052)
3. Low speed refrigerated centrifuge with swinging bucket rotor
4. Ice bucket with cover
5. Flow cytometer (with light scatter detector)

Procedure

Specimen Collection and Preparation of Whole Blood:

Using Vacutainer™ tubes containing EDTA, obtain 5 ml of blood by venipuncture.

(Continued on back page.)

* Patent Pending

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Becton Dickinson Procedures | Direct Immunofluorescence Staining of Cell Surfaces

This method is used to detect cells bearing specific membrane antigens by treating a cell population with monoclonal antibodies conjugated to fluorescein (FITC) or phycoerythrin (PE)*. Direct staining is recommended for analysis by the Fluorescence Activated Cell Sorter (FACS™) or fluorescence microscopy. Direct staining is preferable to indirect staining for the quantitation of antigenic determinants per cell using FACS analysis.

Cells:

1. Mouse or human cell suspension (See Becton Dickinson Procedures on Preparation of Single Cell Suspensions, Sections 2.1 and 2.2).

Reagents:

1. FITC or PE-conjugated monoclonal antibody to human or mouse membrane antigen. Remove aggregates in FITC conjugates by centrifuging at 100,000 x g for 10 minutes immediately prior to use. Do NOT centrifuge PE conjugates.
2. Medium: Phosphate-Buffered Minimal Essential Medium containing 0.1% sodium azide** and 2% calf serum. Store at 2°-8°C. For medium preparation, see Becton Dickinson Procedures on Preparation of Single Cell Suspensions, Section 2.1 and 2.2.
3. Mounting medium, protein free – for microscopy: 90% glycerol in phosphate-buffered saline containing 0.1% sodium azide.

Equipment:

1. Centrifuge fitted with plate carrier
2. Round-bottom microtiter plates or 3 ml plastic tubes
3. Ice bath
4. Fluorescence Activated Cell Sorter (FACS) or fluorescence microscope

Procedure:

1. Dilute 5 μl of FITC or PE conjugated monoclonal antibody in 45 μl medium.

   Add the 50 μl of diluted antibody to microtiter wells or plastic tubes.

2. Adjust concentration of cell suspension to 2 x 10^7 cells/ml. Cells should be > 90% viable as determined by staining with ethidium bromide/acridine orange***(See Becton Dickinson Procedure on Viability Staining using Ethidium Bromide and Acridine Orange, Section 2.3).

   Add 50 μl of the cell suspension (1 x 10^6 cells) to the microtiter wells or plastic tubes and mix gently.

3a. **For staining in microtiter plates,** incubate the mixure for 30–45 minutes on ice. Centrifuge the plate at 200 x g for 3 minutes at 2°-8°C. Carefully aspirate the supernatant from the cell pellet. Wash two times with 100 μl aliquots of cold medium. Centrifuge plate after each washing at 200 x g for 3 minutes. Aspirate supernatant from cell pellet.

3b. **For staining in tubes,** incubate the mixure for 30–45 minutes on ice. Add 2 ml of cold medium, then centrifuge at 300 x g for 5 minutes at 2°-8° C. Carefully aspirate supernatant from cell pellet.

4a. **FACS Analysis:**

Resuspend cells in 0.5 ml of cold medium, to approximately 1 x 10^6 cells/ml. Keep cold until analyzed.

4b. **Fluorescence Microscopy:**

Resuspend cells in 1-2 drops of mounting medium. Deposit a portion of the drop (~ 20 μl) on a microscope slide, place a cover slip over the drop, then seal edge of coverslip with clear nail polish.

Alternatively, prepare cytocentrifuge preparations using Becton Dickinson Method on Immunofluorescence Staining of Cell Surfaces – Cytocentrifuge Preparations, Source Book Section 1.4.

For microscope set-up and filter selection, see *Handbook of Experimental Immunology, 3rd Edition,* pages 15.18–15.21, 1978, ed., Weir, D.M. (Blackwell, Oxford)

* Patent Pending

** **WARNING:** Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compo should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in plumbing where explosive conditions may develop.

*** **CAUTION:** Ethidium bromide and acridine orange are suspected carcinogens. Handle with care.

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Procedure (continued)

Staining Method for Whole Blood:

1. For single color staining, add 5 µl of the antibody per 12 x 75mm tube, for each blood sample. For two color staining, add 5 µl of each antibody per tube.
2. Place 50 µl of whole blood in each tube. Mix gently.
3. Incubate the mixture in the dark in an ice bath for 30 minutes. Remove from ice bath and warm to room temperature.
4. Vortex mixture gently, then immediately add 2 ml of the 1X Lysing Solution to the blood-antibody mixture. Vortex and incubate at room temperature for 3 – 5 minutes. **Note:** Do not extend incubation beyond this time.
5. Centrifuge at 200 x g for 5 minutes.
6. Remove the supernatant by aspiration, leaving ~ 50 µl fluid in the tube to avoid disturbing the pellet.
7. Vortex gently and then add 2 – 3 ml of 1X PBS (or HBSS) to each tube. Vortex again and centrifuge at 200 x g for 5 minutes.
8. Remove the supernatant by aspiration, allowing ~ 50 µl fluid to remain.
9. Add 0.3 – 1.0 ml of appropriate diluent recommended for the flow cytometer. Vortex and add an equal volume of 1% Paraformaldehyde in PBS, vortex and analyze within 48 hours.

Results and Interpretation

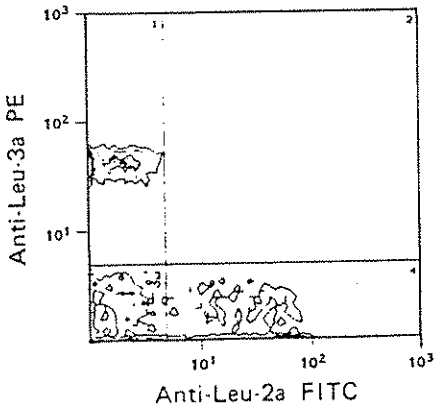
Representative data obtained with the FACS™ Analyzer and the FACS™ 440 Cell Sorter are shown below.

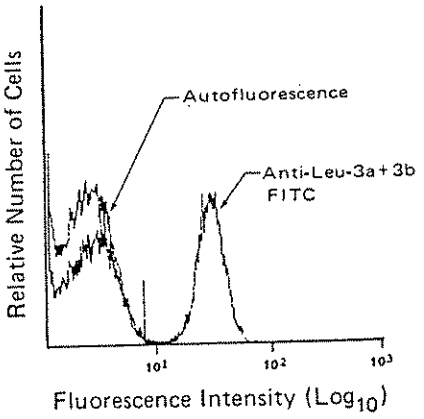
Limitations

1. Each research laboratory should establish normal ranges using its own test conditions.
2. The specimen should contain > 90% viable cells. The cytoplasm of dead cells may stain nonspecifically.
3. The amounts of reagents recommended for use are based on studies of normal human blood.
4. Monoclonal reagents which react with granulocytes (e.g., Anti-Leu-15) will require additional amounts of reagent to saturate all binding sites.

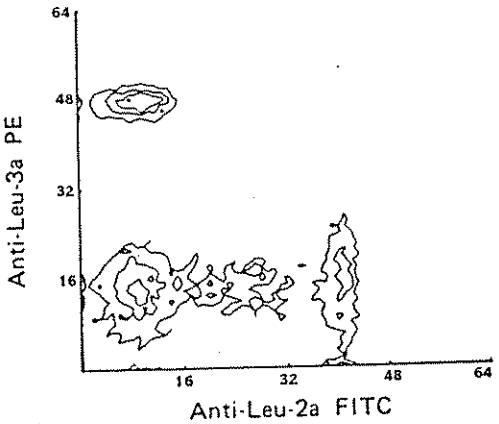
FACS Displays of Peripheral Blood Lymphocytes (Logarithmic Fluorescence Intensity)

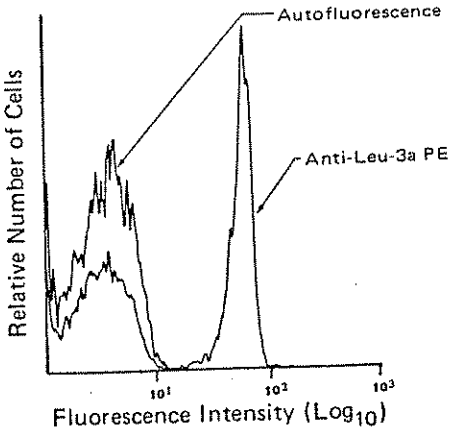
FACS™ Analyzer





FACS™ Cell Sorter





FACS Analysis: Performed on lysed whole blood with gates set on lymphocytes.