Methods | Preparation of the FACS for Aseptic Sorting

Method provided by Ann Cunningham and Dr. Frank Ruddle of Yale University, Department of Biology.

This method is routinely used to prepare the Fluorescence Activated Cell Sorter (FACS II) at Yale for sorting cells for regrowth in culture. While this procedure has been used successfully at Yale, conditions in individual laboratories may require alterations in this protocol to achieve aseptic sorting.

Equipment:

1. Sheath reservoir
2. 0.22 μm filter
3. Sterile, double distilled water
4. Tubing for all lines, cleaned and sterilized
5. Sonicating water bath
6. Sterile 12 x 75 mm tubes
7. 25μ in-line sample filters
8. Sterile 15 ml centrifuge tubes
9. Plastic wash bottles for cleaning nozzles
10. 50 ml syringes with 19 gauge needles

Reagents:

1. 7X™ detergent (Linbro, Flow Labs) – diluted 1/250 sterile distilled water
2. 70% Ethanol
3. Penicillin-streptomycin, 100X (Gibco)
4. Kanamycin, 100X (Gibco)
5. Garamycin (Schering), 50 mg/ml in 10 ml vials
6. Tissue culture medium – Dulbecco's complete Phosphate-Buffered Saline (PBS)
7. 100X $Ca^{++}Mg^{++}$ Stock Solution

   10 g $CaCl_2$
   10 g $MgCl_2 \cdot 6H_2O$

   Dissolve in 1 liter of distilled water. Filter sterilize.
8. Phosphate-Buffered Saline (PBS) Solutions

   10X Stock Solution:

   80 g NaCl
   2 g KCl
   11.5 g Na_2PO_4
   2 g KH_2PO_4

   Dissolve in 1 liter of distilled water. Autoclave.

   1X Working Solution

   50 ml 10X PBS Stock Solution
   5 ml 100X $Ca^{++}Mg^{++}$ Stock Solution
   450 ml sterile distilled water

(Continued on back page)

Becton Dickinson Monoclonal Center, Inc.
2375 Garcia Avenue
Mountain View, California 94043

BECTON DICKINSON

PROCEDURE:

Shut-down. To use the FACS repeatedly for aseptic sorting, clean the instrument lines after every run by filling all lines with 7X. Sonicate the sample filters and nozzles in 7X, rinse in distilled water, then cover and store in sterile tubes with fresh sterile water. Note: excess soaking of nozzles in 7X or ethanol or excess sonication may loosen the jewel.

Start-up. Start running the machine sheath fluid with 7X. Reattach all lines and fill, tapping out bubbles. Run 7X for 30 minutes.

In the meantime, clean the tube holder, collection receptacle, and door with ethanol. After 7X, start an ethanol chase by filling a sample tube with 70% ethanol and driving this through. Stop the sheath fluid flow by closing the sheath valve or clamping the sheath fluid line.

Switch the sheath fluid to sterile PBS. Position a sterile sample tube and back fill the sample lines. Run PBS for 15 - 20 minutes to clear 7X and ethanol. Note: if the lines are not cleared of 7X or ethanol, the drop break-off will be drastically altered.

Weekly Cleanup. Once a week disconnect the tubing and clean it individually -- by pumping the 7X in the long lines and cleaning short lines with a syringe. Rinse all tubing thoroughly and autoclave for 30 minutes. The Yale - Biology group uses Silastic ™ autoclavable tubing (Cole Palmer) for all long lines, and Intramedic ™ tubing for sample delivery lines. Sterilize non-autoclavable tubing by soaking in ethanol and garamycin (50 μg/ml) solutions.

Sonication. Use a small sonicating water bath filled with two inches of distilled water. Insert a beaker filled with water. Sonicate all sorter parts in tubes filled with 7X, held upright in the beaker. Avoid prolonged sonication which produces heat and may loosen the jewel in the nozzle.

Coping with Plugs. If plugging occurs during a sterile run, apply back suction to the nozzle holder to release the plug. (On FACS instruments produced after January 1980, use the Orifice Flush to release the plug). If this does not eliminate the plug, remove the nozzle, clean by sonication, rinse, rinse with ethanol, reattach and continue the run. Once plugging occurs, it is better to completely remove the plug to avoid further trouble. With respect to drop break-off and signal generation it is important to clean it completely. Extensive 7X and ethanol flushes of the lines should remove much of the debris responsible for insidious plugs.

Cell Preparation. It is essential to start with sterile material in order to obtain sterile sorting. Therefore, sterilize all reagents used in cell preparation either by filtration or autoclaving. Plugging problems are likely if the cell sample contains debris, broken cells, or clumped cells. Once DNA from cells is allowed to gel in the nozzle orifice, it is very difficult to remove. Eliminate clumps of cells by passage through nitex cloth with a pore size permitting passage of single cells. For fibroblast cultures, keep in mind that the detached rounded-up cells have a diameter at least three times larger than lymphocytes. Typical cells will be 20 - 30 μ in diameter. Sorting progresses well with an 80 μm nozzle and a cell concentration low enough to minimize coincidental passage.

Cell Collection. Sorted cells can be grown without antibiotics but collection in media containing pen-strep 1X is recommended.

Post-Sorting Treatment. Three to four hours after sorting, wash the cells two to three times with medium containing kanamycin. Wash suspension cells by centrifugation and attached cells by changing medium after the cells have attached. Perform another wash after 24 hours. Grow cells with antibiotics until cultures are definitely sterile, and then use antibiotic-free medium. It is best to save garamycin as a last resort since routine use of powerful broad spectrum antibiotics selects for resistant organisms.

COMPETENT CELLS TB1

1. Innoculate 200 ml of drug free L broth (1:100) from a fresh 5 ml overnight culture.
2. Shake at 37 C to O.D. 550 = 0.2 - 0.3; optimally 0.25 (approx. 1-1.5 hr.).
3. Cool for 10 min. on ice.

   KEEP ON ICE FROM HERE ON

4. Spin cells in sterile 50 ml Falcon tubes at 1.8 - 2.0 x 103 rpm for 15 min. in refrigerated Beckman model J-6B or equivalent.
5. Decant supernatant.
6. Resuspend pellet in 20ml per 50 ml of cells in cold 100 mM CaCl2.
7. Leave on ice 20 min.
8. Spin cells as above.
9. Decant supernatant and resuspend pellet in 2 ml 100 mM CaCl2 plus 14% glycerol per 50 ml tube.
10. Leave on ice at 4 C. (in cold room) 12 - 16 hr., e.g. overnight.
11. Freeze in 0.2 ml aliquots in eppendorfs in dry ice/ethanol bath.
12. Store at -70 C.

QUICK, SMALL, COMPETENT CELL PREP

Do all steps sterile and cold

1. Grow 5ml overnight.
2. Regrow 5mls of a 1:10 dilution in LB broth.
3. Pellet at 5K for 10'.
4. Resuspend in 5mM NaCl (cold).
5. Pellet.
6. Resuspend in 33mM $CaCl_2$.
7. Leave on ice for 20'.
8. Spin down and resuspend in 200µl 33mM $CaCl_2$.
9. Transform.

T. Springer, May 1984

FACS ANALYSIS

I. MAb and FITC-anti-Ig Labeling of Cells. (This protocol is written for rat MAb with mouse cells, but is readily adaptable for other MAb and cell combinations).

A. Reagents

1. Antibodies may be stored as frozen aliquots and de-aggregated prior to each use by Beckman microfuge centrifugation x 5 min.

Storage: MAb: -20 to -35° for IgG or -80° for IgM.
FITC anti-Ig: -80°.

This works well for MAb and for our standard FITC-rabbit $F(ab')_2$-anti-rat IgG reagents.

The alternative procedure is to deaggregate antibodies by centrifugation, dilute the desired amount in L15 HGB, then millipore filter and store sterile aliquots at $+4^{\circ}$. This is advisable for many fluorochrome-coupled reagents, especially IgM's which are prone to aggregate or lose activity upon freezing.

2. MAb are usually used undiluted as MAbCS (MAb culture supernatants), but some MAbCS can be diluted as much as 10^{-2} without appreciable drop in FI (fluorescence intensity).

Dispense 50 µl aliquots into V well microtiter plates. Plates may be stored on ice up to 24 h before use.

3. Affinity-purified FITC-rabbit $F(ab')_2$ anti-rat IgG, absorbed with mouse IgG, is our standard second reagent. Use sparingly, as this is an extremely precious reagent and much work went into its preparation (TS)*. Last steps are described and earlier steps referenced in TS 29 59. This particular batch is 0.48 mg/ml, F/P 19 µg/mg. Use at 1/16 dilution, 50 µl per sample. This gives essentially saturation labeling of H-2 or Ia on spleen cells. Labeling which is proportional to first antibody site number is often desirable, but saturation is unnecessary. Proportionality should be met if the FITC-antibody is not depleted for the most dense antigen, i.e. if double the volume of FITC anti-IgG at the same dilution gives less than a 10% increase in fluorescence intensity (FI). For high density (e.g., H-2 or Thy-1) antigens on tumor cells, (which have more antigen/cell) higher concentration should be used only if proportional labeling is required (use 1/8). It would be interesting to test FITC-MAb to rat IgG as second reagents.

B. Wash and Cell Suspension Media

L15 HGB (L15 HG + 0.5% BSA)
To 500 ml L15 HG (L15 medium + 10 mM HEPES + glucose) add 8.3 ml 30% BSA. Use for cell preparation.

* Affinity purified, FITC conjugated antibodies are now

available commercially, e.g. Cappel Labs, Malvern, PA and Zymed, South San Francisco, CA.

L15 HGBA (L15 HGB + 0.01 M $NaNa_3$): To 200 ml L15HGB add 2.6 ml 5% NaN_3 or 13.3 ml 0.15 M NaN_3. Use for diluting fluorochrome reagents, for washes, and for final cell suspension.

For last suspension of cells before adding to MAb use L15HGB + 57 mM HEPES, prepare 20 ml. The higher buffer concentration moderates the bicarbonate in MAbCS.

C. Protocol

The protocol is designed for use with microtiter plates to allow 60 samples or more to be analyzed at one time. It can be modified for use in 1.5 ml microfuge tubes.

1. All steps and reagents are held at 4^{0} or on ice. Antibodies should be deaggregated, and diluted and aliquoted the day before to allow labeling to be completed in morning (50 µl/well).

2. Prepare cell suspensions in L15 HGB, dilute to ~ 12 ml and centrifuge. It is best not to Ficoll-Hypaque purify, since selective B cell enrichment can occur if cell recoveries are low.

3. Suspend in a small volume of L15 HGB + 57 mM HEPES and count.

4. Dilute to 2.5×10^{7} viable white cells/ml.

5. Add 50 µl cells/well. Place on microshaker II, pulse 1 min at top speed at 0, 10, and 20 min. 30 min total.

6. Wash 3x with L15 HGBA, centrifuging at 200 g x 5 min.

7. Suspend cells in 50 µl fluorochrome reagent by 1 or 2 up and down pipettings. Shake every 10 min as above, total incubation of 30 min.

8. Wash 3x.

9. Suspend cells in 0.2 ml/well, and filter through 1-1/4'' x 1-1/4'' square of 37 µ nytex (Small Parts No. CMN-37) and into capless Fisher No. 14-959-12B tubes, washing through with 1 ml L15 HGBA.

10. Normally cells are analyzed the same day. They may be stored on ice O/N for analysis the next AM or formaldehyde-fixed, but only with some loss of resolution, particularly of light-scattering differences between populations as in bone marrow.

D. Controls

1. Use NSI CS + 50 µg/ml normal rat IgG <u>or</u> M1/69.16.11 HK MABCS as control first antibodies for each different cell type.

2. If labeling proportional to site number is important, include one control utilizing 2-fold higher FITC-anti-IgG volume with the cell and MAb combination expected to give brightest labeling.

3. Glutaraldehyde-fixed sheep red blood cells, stored at $-80^{\circ}C$ in small aliquots, are used to calibrate the FACS.

4. B6/J spleen cells labeled with the M1/42 anti-H-2 MAb can serve as a standard for comparing FI in different experiments.