



Flow Cytometry of ESCs

Background:

For characterization, a single cell suspension of the cell line in question is analyzed for the presence of specific antibody markers by flow cytometry. The markers were selected from a pool of antibodies based on their availability and their specificity to undifferentiated stem cells and the differentiated cells in each germ layer (ectoderm, mesoderm, and endoderm). The antibodies in the following protocol are used to characterize mouse embryonic stem cells (ESC). Other antibodies are recommended to analyze human ESC.

Antibodies:

Primary Antibodies	Source	Dilution
Oct-3/4	BD Transduction Laboratories	1:250
SSEA1	Chemicon	1:100
EMA1	Developmental Studies Hybridoma Bank	1:10
MF-20	Developmental Studies Hybridoma Bank	1:500
Troma	Developmental Studies Hybridoma Bank	1:100
GFAP	BD Biosciences Pharmingen	1:500
NeuN	Chemicon	1:120

Secondary Antibodies	Source	Dilution
Alexa 488 Goat anti-mouse	Molecular Probes	1:750
Alexa 488 Goat anti-rat	Molecular Probes	1:750

Procedure:

1. Trypsinize cells and collect in a 15-ml tube.
2. Spin down the cells and resuspend in growth medium.
3. Perform a cell count
4. Fix cell suspension with paraformaldehyde:
 - a. Spin down and wash with 10 ml of 1X PBS without Ca and Mg (ATCC® SCRR-2201).
 - b. Resuspend the pellet in 10 ml 2% paraformaldehyde solution:
 - 1 ml 20% paraformaldehyde
 - 1 ml 10X PBS
 - 8 ml ultrapure water
 - c. Incubate at room temperature for 20 minutes.
 - d. Spin down and resuspend pellet in 10 ml of 1% normal goat serum (NGS) in 1X PBS.
5. Wash paraformaldehyde-fixed cells 2 times in 1% NGS.
6. Resuspend the cells in a volume of 3% NGS necessary for a concentration of 2×10^6 cells/ml, based on the cell count in step #3. You will need $\sim 1 \times 10^6$ cells per test condition.
7. Label 1.5-ml BSA-coated microcentrifuge tubes as below and place into a microcentrifuge tube rack.
 - a. Intracellular Staining (I)
 - i. Unstained, I
 - ii. Mouse 2ab, I
 - iii. Rat 2ab, I

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- iv. Oct-3/4, I
 - v. Troma, I
 - vi. GFAP, I
 - vii. MF-20, I
 - viii. NeuN, I
 - b. Surface Staining (S)
 - i. Unstained, S
 - ii. Mouse 2ab, S
 - iii. SSEA1, S
 - iv. EMA1, S
 8. Discard the BSA solution from each 1.5 ml tube.
 9. Add 0.5 ml of the cell suspension to each 1.5-ml tube (~1 x 10⁶ cells).
 10. Centrifuge all 1.5-ml tubes at 960 to 1000 x g for 3 minutes at room temperature.
 11. Aspirate off the supernatant from each tube carefully.
 12. Add blocking solution and/or detergent to the tubes and resuspend the pellet.
 - a. Intracellular: 150 µl 3% NGS in 1X PBS + 150 µl 1% saponin
 - b. Surface: 300 µl 3% NGS in 1X PBS
 13. Incubate at room temperature for 15 minutes.
 14. Spin down all Intracellular tubes, remove the saponin solution, and resuspend in 300 µl of 3% NGS.
 15. Incubate all tubes for 15 more minutes at room temperature.
 16. Centrifuge tubes to pellet cells.
 17. Carefully aspirate the supernatant.
 18. Add 300 µl of primary antibody and resuspend the pellet. Add 300 µl of blocking solution to the unstained and secondary controls.
 19. Incubate tubes for 1 to 2 hours at room temperature.
 20. Centrifuge tubes to pellet cells.
 21. Wash 3 times with 1% NGS. (This does not apply to unstained and M2ab tubes.)
 22. Add 300 µl of the correct secondary antibody to each tube and resuspend the pellet. Do not add antibody to the unstained S control.
 23. Incubate tubes for 0.5 to 1.0 hours at room temperature in the DARK. It is critical to keep the samples in a drawer or wrapped in foil away from light.
 24. Centrifuge tubes to pellet cells.
 25. Wash 3 times with 1% NGS.
 26. Add 0.5 ml of 1X PBS to each tube and resuspend the pellet.
 27. Transfer the resuspended pellet from each 1.5-ml tube to a labeled flow cytometry tube. Protect the samples from light.
 28. Perform flow cytometry using a FACSCalibur (BD Biosciences) flow cytometer according to manufacturer's instructions.

Data Acquisition: A BD FACSCalibur flow cytometer with a dual laser source (488 and 635 nm) is used to acquire data. We have been using one parameter staining (FITC only). Unstained cells and cells with only secondary antibody are used to adjust photomultiplier voltages in order to localize background autofluorescence to the lower left hand quadrant of dot-plot display.

Gating: The process of gating facilitates an accurate analysis of the data acquired. It is necessary to perform gating for the following reasons:

1. Embryoid bodies and undifferentiated stem cell preparations may contain large clumps of cells even after trypsinization. These clumps must be excluded from the analysis.
2. A preparation of ESCs that have been cultured on a feeder layer contain both types of cells after trypsination. STO and MEF cells are larger than mouse ESCs and are easily excluded from flow cytometric analysis by gating.

Data Analysis:

Data analysis is done using CellQuest software (BD Biosciences) installed on a Macintosh computer.

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