

## Immunocytochemistry of Embryoid Bodies

### Background

For characterization, embryoid bodies (EBs) cultured in vitro are analyzed for the presence of specific antibody markers by immunocytochemistry. 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 embryonic stem cells.

### Suggested 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

- Twenty-four hours before staining, aliquot EBs into individual wells of a 4-well Lab-Tek chamber slide (Nunc). You will want 10 to 12 EBs per well for each antibody and control.
- Add 0.75 ml of ESC growth medium without leukemia inhibitory factor (LIF) to each well.
- Incubate at 37°C with 5% CO<sub>2</sub> for 24 hours.
- Carefully pipette off the ESC growth medium in each well.
- Wash each well gently with 0.5 ml of 1X PBS without Ca and Mg (ATCC® SCRR-2201).
- Add 0.5 ml of 4% paraformaldehyde solution to each well. (Gloves should be worn and care taken when working with this chemical. See MSDS.)  
2 ml 20% paraformaldehyde
- 1 ml 10X PBS  
7 ml dH<sub>2</sub>O
- Incubate at room temperature for 20 minutes.
- Pipette off the 4% paraformaldehyde.
- Carefully wash the wells 3 times with 0.5 ml of 1X PBS.
- Add 0.5 ml of appropriate blocking solution to each well. For intracellular staining, add 0.5 ml of appropriate blocking solution containing 1% Triton to each well.  
Note: The blocking solution should be 3% serum in 1X PBS. The type of serum should match the host of the secondary antibody to be used. For example, 3% normal goat serum (NGS) is used when goat anti-mouse is the secondary antibody.
- Incubate at room temperature for 1 hour.

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12. Pipette off the blocking solution.
  13. Add 0.3 ml of primary antibody. The primary antibody should be diluted to the proper working concentration with 3% NGS in 1X PBS (ATCC<sup>®</sup> 30-2201).
  14. Incubate at room temperature for 2 hours.
  15. Pipette off the primary antibody.
  16. Carefully wash the wells 3 times with 0.5 ml of 1% NGS in 1X PBS.
  17. Add 0.3 ml of secondary antibody. The secondary antibody should be diluted to the proper working concentration with 3% NGS in 1X PBS. Secondary antibody dilutions for intracellular primary antibodies should include 0.1% Triton.
  18. Incubate at room temperature without light (in a drawer or wrapped in foil) for 1 hour.
  19. Pipette off the secondary antibody.
  20. Carefully wash the wells 3 times with 0.5 ml of 1% NGS in 1X PBS.
  21. Remove excess liquid.
  22. Carefully separate wells and gasket from slide by following the directions provided on the package. Discard wells and gasket.
  23. Mount one larger rectangular coverslip or two small square coverslips onto each slide using a couple drops of fluorescence-compatible mounting medium. Try to avoid creating bubbles.
  24. View the slides immediately or place them in a container and wrap it with foil. Store the slides at 4°C.

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