

Immunocytochemistry of ESCs

Background:

For characterization, cells cultured in vitro on glass coverslips of the cell line in question are analyzed for the presence of specific antibody markers by immunostaining. The markers were selected from a pool of antibodies based on their availability and their specificity to embryonic stem cells. The antibodies used in this protocol are selected for mouse embryonic stem cells (ESC). Other antibodies are recommended for 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

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

Cell Culture:

- In sterile culture hood, place one sterile glass coverslip in the appropriate number of wells in a 24-well culture plate, using forceps or suction with vacuum and sterile serological pipette.
- Label the wells according to the conditions to be used. (i.e., unstained, mouse secondary, Oct-3/4)
- Thaw CF-1 mouse embryonic fibroblasts (MEF) and plate $\sim 2.5 \times 10^5$ MEF per well.
- Allow the MEF cells to incubate 24 hours in an incubator (5% CO₂ at 37°C, humidified).
- Remove the MEF growth medium from the well containing the glass coverslips.
- Thaw and plate ESCs at approximately 75,000 per well and allow them to grow for 1 to 2 days.
- Remove the DMEM-F12 growth medium from the wells containing the cultured cells on glass coverslips by using an aspirator or pipetting.
- Wash each well with 1.0 ml of 1X PBS at room temperature.
- Remove the PBS wash and add 1.0 ml 2% paraformaldehyde solution:
1 ml 20% paraformaldehyde
1 ml 10X PBS
8 ml ultrapure water
- Incubate the samples for 20 minutes at room temperature.
- Remove the fixative by using an aspirator or pipetting. Note: The fixed cells may be stored at 4°C in 1X PBS solution for up to one week prior to immunostaining.

Immunostaining:

1. Add 1.0 ml of 1x PBS to rinse each well of the 24 well plate of paraformaldehyde fixed cells; rinse briefly at room temperature.
2. Remove PBS from each well and add blocking solution and/or detergent to wells accordingly.
Intracellular: 300 μ l of 0.5% saponin
Surface: 300 μ l of 3%NGS in 1X PBS.
3. Incubate at room temperature for 15 minutes.
4. Aspirate wells containing saponin solution.
5. Add 300 μ l of 3% NGS in 1X PBS to the wells from which the 0.5% saponin was removed.
6. Incubate all wells for 45 more minutes at room temperature.
7. Carefully aspirate off the supernatant.
8. Add primary antibody to labeled wells: Using correct working dilutions, add 300 μ l of the specified primary antibody to each well. Leave 300 μ l of 3% NGS in 1X PBS in the unstained and secondary control wells.
9. Incubate tubes for 1 to 2 hours at room temperature.
10. Remove the primary antibody solutions from the wells.
11. Wash the cells 3 times with 1% NGS in 1X PBS.
12. Add secondary antibody to labeled wells.
Using the correct secondary antibody and working dilution, add 300 μ l secondary antibody to each well. Leave 300 μ l of 1% NGS in 1X PBS in the unstained control wells.
13. 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.
14. Wash the cells 3 times with 1.0 ml of 1% NGS in 1X PBS.
15. Remove the glass coverslips from the wells using forceps and place the coverslips, cell-side down, on an appropriately labeled microscope slide containing 1 to 2 drops of mounting medium.
16. Visualize the immunostained cells/colonies under an epifluorescence microscope and record images.
17. Store microscope slides with coverslips at -20°C in the dark.

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