Expansion and Mitomycin C Treatment of Fibroblast Cells

I. Expanding Untreated Fibroblasts:
Untreated fibroblasts are expanded until confluent and then inactivated (either by mitomycin C or irradiation) when used as feeder cells to support the growth of human or mouse embryonic stem (ES) cells. GlobalStem strongly recommends not expanding beyond passage five (P5). Otherwise, the capacity to support ES cell growth may be compromised.

All medium and reagents used in the culture of this product should be warmed to 25-37°C before use.

1. Place the frozen vial into a 37°C water bath as soon as possible and retrieve the vial before the contents are completely thawed (approx. 1 min.).
2. Immediately transfer the contents of the vial to a 15-mL tube and dilute 1:10 with complete growth medium.
3. Spin the cell suspension at 270 x g for 5 minutes in order to pellet the cells.
4. Resuspend the pellet in complete growth medium and plate the cells to the appropriate size tissue culture flask. The typical plating density for propagation is 13,000–18,000 cells/cm².
5. In 4–5 days when the culture is confluent, the flask can be subcultured at a 1:4 ratio using 0.25% trypsin/EDTA. Before adding trypsin, add sufficient volume of 1X PBS to remove any proteins that may inhibit trypsin activity. Aspirate the PBS and add an adequate amount of trypsin to cover the cell growth area entirely. The cells should detach from the surface in 1–2 minutes.
6. Inactivate the trypsin with an equal volume of complete growth medium and pipet the cells to a single cell suspension.
7. Transfer the cell suspension to a tube and rinse the flask once to collect any remaining cells.
8. Spin the cell suspension at 270 x g for 5 min. in order to pellet the cells.
9. Resuspend the cells and add to flasks for further expansion.
II. Mitomycin C Treatment of Fibroblast:
Inactivated primary MEFs are used to promote growth and prevent differentiation of embryonic stem cells. MEFs can be inactivated either by chemical treatment or irradiation. When access to an irradiator is limited, treatment with mitomycin C is recommended.

*Care must be taken when handling mitomycin C as it is genotoxic. Wear gloves, sleeve, and a lab coat when working with the substance. Solutions containing this chemical should be disposed of according to the MSDS.*

1. Reconstitute mitomycin C (Calbiochem #475820) to make 1 mg/mL mitomycin C solution. Mix thoroughly.
2. Add the necessary amount of 1 mg/mL mitomycin C solution to flasks containing 80-90% confluent fibroblasts and MEF culture medium to achieve a 10 ug/ml final concentration (i.e., 400 μl into 40 ml).
3. Incubate the flasks for 2.5–3 hrs at 37°C in humidified incubator with 5% CO₂.
4. Carefully remove culture medium containing mitomycin C and store in a designated waste bottle for proper disposal.
5. Wash each flask with 10 mL PBS. Remove the wash and add it to the mitomycin C waste bottle.
6. Wash again with 10 mL PBS.
7. Add appropriate amount of 0.25% trypsin-EDTA to the flask and incubate for 1 min. in a humidified, 37°C incubator with 5% carbon dioxide.
8. While tapping the flask, observe the cells under an inverted microscope until cells detach (1.5–2 min.).
9. Add equal amount of culture medium to inactivate the trypsin and rinse surface of the flask to detach all cells. Gently pipetting up and down will break cell clumps.
10. Transfer the cell suspension into a centrifuge bottle or tube. Wash the flask with medium.
11. Centrifuge at 270 × g for 5 min. Discard the supernatant.
12. Resuspend the pellet in the culture medium.
13. Perform cell count and dilute the cell suspension to twice the final concentration. The final concentration can be up to $12 \times 10^6$ cells/vial.

14. Add an equal volume of cold 2X freezing medium to the cell suspension.

15. Aliquot 1 mL of the suspension into each vial.

16. Place the vials in an appropriate insulated freezing container and place in a $80^\circ$C freezer overnight. The next day transfer the vials into liquid nitrogen.