Technical Notes—How to Passage and Subculture—
Fetal Liver Stromal Cells

Thawing and Culturing Cells

1. Calculate the number of flasks needed. The recommended seeding density for AllCells FL-Stromal cells is 5,000 to 10,000 cells/cm^2. For example, one vial of AllCells Stromal Cells ($7.5x10^5$/vial) is sufficient for one to two T75 flasks or three to six T25 flasks.

2. Prepare the culture media:

   a. Add 2mM concentration of L-glutamine and the entire contents of 50ml AllCells Fetal Stromal Stimulatory Supplement (Cat. No. FL-003) to 450ml of AllCells Basal Medium (Cat. No. FL-002).

   b. Add 2-3 ml/5 cm^2 of the mixed media into the T75 or T25 flasks.

   c. In a 37°C, 5% CO_2 humidified incubator, place the media in flasksto equilibrate in the incubator for at least 30 minutes.

3. Wipe the frozen vial with 70% alcohol before thawing. In a biosafety hood, briefly twist the cap a quarter-turn to relieve pressure, and then retighten the cap.

4. In a 37°C water bath, quickly thaw the vial. Be careful not to submerge the entire vial in the water bath. Do not remove the vial until a tiny ice-crystal is left. Wipe the outside of the vial with 70% alcohol.

5. Count the cells using *Trypan Blue Method*. In a biosafety hood, take a 10ul sample from the vial. Mix the 10ul sample with 10µl of trypan blue. Dilute the cells if necessary and count the number of cells on a hemacytometer to determine the viability.

N = # of cells counted on all 4 squares of a hemacytometer
d= dilution factor

Trypan Blue Method Equation for Cell Count:
# of cells/ vial =N/4 x 2x d x_____ ml = _____10^4

Equation for Viability:
# of cells excluded by trypan blue/ total #of cells x 100% = ______%

**This is a very important step to determine if the cell number viability matches what AllCells claims.

Maintaining Cell Cultures

1. Change the growth medium the day after seeding and every other day thereafter.

2. In a sterile container, warm an appropriate amount of medium to 37°C.

3. Remove the medium from the culture flasks and replace with the warmed, fresh medium; return the flask to the incubator.

Note: Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer only the required volume to a sterile secondary container.

Technical Notes—How to Passage and Subculture—Fetal Liver Stromal Cells (continued)

Subculturing Cells

1. Subculture the cells when they are 70 to 80% confluent (about 10 days). Many mitotic figures should be visible throughout the flask.

2. For each 25cm^2 of cells to be subcultured: (1) Prepare 2 ml of Trypsin/EDTA, 5 ml of HEPES Buffered Saline Solution (HEPES-BSS), 4 ml of Trypsin Neutralizing Solution (TNS such as 10% FBS-DPBS). Allow the aforementioned items to come to room temperature; (2) From one culture flask, remove the medium and rinse the flask with 5 ml of room temperature HEPES-BSS. (This is an IMPORTANT step. The culture medium contains complex proteins that neutralize the effect of trypsin.) Remove the HEPES-BSS from the flask; (3) Repeat step 2 for each culture flask.

3. Add 2 ml of Trypsin/EDTA solution to each T25 flask. Allow the trypsinization to continue until approximately 90% of the cells have lifted up from the T25 flask. This entire process takes about 2 to 6 minutes.

4. Hit the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, wait 30 seconds and repeat this motion again. If cells still do not detach, wait and repeat every 30 seconds thereafter.

5. After cells have detached, neutralize the trypsin in the flask with 4 ml of room temperature Trypsin Neutralizing Solution.

6. If the majority of cells do not detach within 7 minutes, the trypsin is either not warm enough or not active enough to release the cells.

7. Harvest the culture vessel as described above, either retrypsinize with fresh, warm Trypsin/EDTA solution or rinse with Trypsin Neutralizing Solution, then, add fresh, warm medium to the culture vessel, and return to an incubator until fresh trypsinization reagents are available.

8. Quickly transfer the detached cells to a sterile 15ml centrifuge tube and rinse the flask with a final 2 ml of HEPES-BSS to collect residual cells.

9. Add this rinse to the centrifuge tube.

10. Under the microscope, examine the harvested flask to make sure the harvest was successful. Look at the number of cells left behind. This number should be less than 5%.

11. Centrifuge the harvested cells at 220 x g for 5 minutes to pellet the cells.

12. Dilute the cells in 4 to 5 ml of growth medium and count the cells with a hemacytometer.

13. Culture the cells. (Refer to the *Thawing and Culturing Cells*)