

Technical Notes—How to Passage and Subculture— Marrow Stromal Cells

Thawing and Culturing Cells

1. Calculate the number of flasks needed. The recommended seeding density for AllCells Marrow Stromal Cells (MSC) is 5,000 to 10,000 cells/cm². For example, one vial of AllCells MSC (7.5x10⁵/vial) would be sufficient for 1 to 2 T75 flasks or 3 to 6 T25 flasks.
2. Prepare the culture media:
 - a. Add 2mM concentration of L-glutamine and the entire contents of 50ml AllCells Stimulatory supplement (Cat. No.MSC-003) to 450ml of AllCells Basal Medium (Cat. No. MSC-002).
 - b. Add 2 to 3 ml/5 cm² of the mixed media into the T75 or T25 flasks.
 - c. In a 37°C, 5% CO₂ humidified incubator allow the flasks to equilibrate in 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, 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. In a biosafety hood, take a 10ul sample from the vial. Mix the 10ul sample with 10ul 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

Equation for Cell Count:

$$\# \text{ of cells/ vial} = N/4 \times 2 \times d \times \text{ml} = \text{ } 10^4$$

Equation for Viability:

$$\# \text{ of cells excluded by trypan blue/ \# total number of cells} \times 100\% = \text{ } \%$$

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

Maintaining Cell Culture

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 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.

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 25cm² of cells to be subcultured:
 - a. 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.
 - b. 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.
 - c. Repeat Step b 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 two to six minutes. 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.
4. After cells have detached, neutralize the trypsin in the flask with 4 ml of room temperature Trypsin Neutralizing Solution. 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. Harvest the culture vessel as described above, and either re-trypsinize 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.
5. 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. Add this rinse to the centrifuge tube.