

# Technical Notes—How to Passage and Subculture—Normal Human Dermal Fibroblasts

## Recommended Tissue Dissociation Reagent

- HEPES Buffered Saline Solution (HEPES-BSS), 1X, Room Temperature
- Neutral Proteases, such as Dispase (sometimes marketed as Accutase) or Thermolysin
- NHNDF Basal Medium, 450ml, not supplemented (Cat#: HN006006)
- NHNDF Supplement, 10X, 50ml (Cat#: HN006007)

Table 4: Reference For Fibroblast Cell Culture

Types of Culture Vessels	Number of Cells That Can Be Seeded	Amount of Culture Media
T-25 flask	8.75x10 <sup>4</sup>	5mL
T-75 flask	2.6x10 <sup>5</sup>	15mL
T-150 flask	5.25x10 <sup>5</sup>	25mL
T-225 flask	7.87x10 <sup>5</sup>	40mL

**Note:** Do not to leave the fibroblast cells thawed for too long before culturing.

## How to Thaw Cells and Set Up Culture

1. Calculate the number of flasks needed based on the cell number
  - a. The recommended seeding density for AllCells NHNDF is 3,500 cells/cm<sup>2</sup>.
  - b. The cell number can be found on the Certificate of Analysis.
2. Make the NHNDF culture medium by diluting the NHNDF supplement (Cat#: HN006007) at a ratio of 1:10 in the NHNDF Basal Medium (Cat#: HN006006).
3. Place recommended amount of culture medium in the desired culture flask.
4. In a 37°C water bath, warm a few extra milliliters of supplemented medium to dilute the thawed cells (about 7ml of medium is enough for a 1.8ml frozen vial).
5. 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 re-tighten the cap.

6. In a 37°C water bath, quickly thaw the vial. Be careful not to submerge the entire vial in the water bath.
7. Thaw the content of the vial as quickly as possible, and remove the vial when only a tiny ice-crystal is left. If vials are left in the 37°C water bath for longer than 2 minutes, the cells can suffer serious damage. Wipe the outside of the vial with 70% alcohol.
8. Transfer the thawed cell suspension into a sterile 15ml or 50ml centrifuge tube, and add 7ml of the 37°C culture medium to the cells drop-wise while gently mixing the cells.
9. Take a small sample from the dilution for cell count, calculate the exact number of cells and dispense, according to the recommended seeding concentrations into the corresponding culture flasks with the correct amount of media (refer to Table4). Gently rock the culture flasks back, forth, and then sideways to evenly distribute the cells and return to the incubator. Do not swirl the flask in a circular motion as this will concentrate the cells in the middle of the flask.

**Note:** It is not necessary to wash the cells; the effects of washing can induce more harm to the cells than the effects of DMSO residue in the culture.

## Maintaining Cell Culture

1. Every two to three days, feed the NHNDF cell culture by completely replacing the culture medium with fresh NHNDF supplemented medium.
2. Aliquot the appropriate amount of NHNDF medium needed per flask (refer to Table 4) into a sterile container, and warm medium to 37°C inside a 37°C water bath. Remove the flask from the 37°C, 5% CO<sub>2</sub> incubator, gently aspirate and discard the culture medium from the flask, and replace the medium with fresh, 37°C supplemented NHNDF culture medium.
3. Return the flask to the incubator and continue culture.

**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—Normal Human Dermal Fibroblasts (continued)

## Subculturing Cells

1. Subculture the cells when they have reached approximately 80% confluent in monolayer (about six to seven days).
2. Clean all reagent containers surfaces with 75% ethanol use to avoid contamination.
3. Thaw the 50ml 10X NHNDF Supplement and add to the 450ml un-supplemented NHNDF basal medium.
4. Leave the supplemented NHNDF medium inside the hood to equilibrate to room temperature.
5. Thaw other frozen reagents, such as the neutral protease, and allow it to equilibrate to room temperature.
6. Prepare the flasks:
  - a. From one culture flask, remove and discard the medium with a pipet.
  - b. Add 5 ml of room temperature HEPES-BSS.
  - c. Cap the flask and gently rock the flask back and forth and sideways. (This is an IMPORTANT step. The culture medium contains complex proteins that can neutralize the effect of proteases.)
  - d. Remove and discard the HEPES-BSS solution from the flask.
  - e. Repeat the HEPES-BSS wash once for each culture flask.
7. Add the appropriate amount of the neutral proteases, following the manufacturer's recommendation for different flask sizes.
8. Gently rock the flask to spread the protease solution, replace the cap, and allow the flask to sit in the hood for about 2-3 minutes.
9. Using an inverted microscope, look at the flask to observe the magnitude of detachment.
10. After cells have detached, neutralize the protease with the supplemented NHNDF culture medium. Add 2X v/v (volume:volume) of the NHNDF medium. (For example, 4ml of the NHNDF medium for every 2ml of protease solution used) Mix the entire cell suspension well and use it to rinse any residual cells off the culture surface and walls of the flask.
11. Transfer the detached cells to a sterile 15ml or 50ml centrifuge tube and rinse the flask with 5-10ml of NHNDF culture medium to collect more residual cells. Add this rinse to the centrifuge tube.
12. Examine the flask on an inverted microscope to make sure the harvest was successful. If residual cells cover more than 5% of the viewing field, repeat **Steps 6 through 10** until less than 5% of the viewing field is covered with cells.
13. Centrifuge the harvested cells at 220g for 5 minutes to pellet the cells.
14. Discard the supernatant and re-suspend the cells in 2 to 3ml of the NHNDF culture medium and count the cells with a hemacytometer.
15. Set up flasks needed based on the cell number you would like to seed per flask and the flask size. The recommended seeding density for NHNDF is 3,500 cells/cm<sup>2</sup>. (Refer to Table 4)
16. Seed the cells at the correct density in the appropriate flasks.
17. Please refer to "Maintaining Cell Culture" section on page 196 for culture process.

**Note:** Neutral proteases are effective for cell dissociation; they are much gentler on the cell membrane and work quickly at room temperature. Cells that are already detached can wait several minutes longer until the remaining cells are completely released.