

Technical Notes—How to Passage and Subculture—Normal Human Neonatal Epidermal Keratinocytes

General Media Requirements

- Recommended Thawing and Cell Passaging Reagents HEPES Buffered Saline Solution (HEPES-BSS), 1X, Room Temperature
- Neutral Proteases, such as Dispase (sometimes marketed as Accutase), or Thermolysin
- NHNEK Basal Medium, 500ml, not supplemented (Cat#: HN007006)
- NHNEK Supplement Kit, 5ml (Cat#: HN007007)

If a strictly serum-free condition is not required, IMDM (Iscove's Modified Dulbecco's Medium) or RPMI-1640 media supplemented with 10% FBS (fetal bovine serum) can be used to thaw the frozen cells or washing cultured cells.

Table 5: Reference For Keratinocyte Culture

Types of Culture Vessels	Recommended Number of Seeding Cells	Amount of Culture Media
T-25 flask	1x10 ⁵	5mL
T-75 flask	3x10 ⁵	15mL
T-150 flask	6x10 ⁵	25mL
T-225 flask	9x10 ⁵	40mL

Thawing and Culturing Cells

Do not leave keratinocytes thawed for an extended amount of time before culturing.

1. Calculate the number of flasks needed based on the cell number.
 - a. The recommended seeding density for AllCells Normal Human Neonatal Epidermal Keratinocytes (NHNEK) is 4,000 cells/cm².
 - b. The actual product cell number can be found on the Certificate of Analysis.
2. Make the NHNEK culture medium by diluting the NHNEK supplement (Cat#: HN007007) in the NHNEK basal medium (Cat#: HN007006).

3. Place recommended amount of culture medium in the desired culture flask.
4. In a 37°C water bath, warm a few 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. Remove the vial when only a tiny ice-crystal is left. If vials are left in the 37°C water bath for longer than two minutes, the cells can suffer serious damage.
7. Wipe the outside of the vial with 70% alcohol.
8. Transfer the thawed cell suspension into a sterile 15ml or 50ml conical tube.
9. While mixing the cells gently, add 7ml of the 37°C culture medium to the cells drop-wise.
10. 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 **Table 5**). Gently rock the culture flasks back and 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.
11. After the first 24 hours of incubation, remove all of the media from the flask and replace the flasks with new, supplemented culture media.

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

Technical Notes—How to Passage and Subculture—Normal Human Epidermal Keratinocytes (continued)

Maintaining Cell Culture

1. Every two to three days, feed the NHNEK cell culture by completely replacing the culture medium with fresh NHNEK supplemented medium.
2. Aliquot the appropriate amount of NHNEK medium needed per flask (refer to Table 5) into a sterile container, and warm medium to 37°C inside a 37°C water bath.
3. Remove the flask from the 37°C, 5% CO₂ incubator, gently aspirate and discard the culture medium from the flask, and replace the medium with the fresh, 37°C supplemented NHNEK culture medium.
4. Return the flask to the incubator and continue culture.

Note 1: Avoid repeated warming and cooling of the NHNEK medium. If the entire contents are not needed for a single procedure, transfer only the required volume to a sterile secondary container. Protect the NHNEK medium from light.

Subculturing Cells

1. Subculture the cells when they have reached approximately 80% confluent in monolayer (usually about 5-6 days). Many mitotic figures should be visible throughout the flask.
2. Clean all reagent containers surfaces with 75% ethanol use to avoid contamination.
3. Thaw the NHNEK Supplement and add it to the un-supplemented NHNEK basal medium.
4. Thaw other frozen reagents, such as the neutral protease, and allow all to equilibrate to room temperature.
5. 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.

6. Add the appropriate amount of the neutral proteases, following the manufacturer's recommendation for different flask sizes. 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. Using an inverted microscope, look at the flask to observe the magnitude of detachment.

Note 2: 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.

7. After cells have detached, neutralize the protease with the supplemented NHNEK culture medium. (Please see Note 1.) Add 2X v/v (volume:volume) of the NHNEK medium. (For example, 4ml of the NHNEK medium for every 2ml of protease solution used.) Mix the entire cell suspension well and use the cell suspension to rinse any residual cells off the culture surface and flask walls.
8. Transfer the detached cells to a sterile 15ml or 50ml centrifuge tube and rinse the flask with 5 to 10ml of NHNEK culture medium to collect more residual cells. Add this rinse to the centrifuge tube.
9. 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 3 through 9 until less than 5% of the viewing field is covered with cells.
10. Centrifuge the harvested cells at 220g for 5 minutes to pellet the cells.
11. Discard the supernatant and re-suspend the cells in 1-2ml of the NHNEK culture medium and count the cells with a hemacytometer.
12. 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 AllCells NHNEK is 4,000 cells/cm². (Refer Table 5)
13. Seed the cells at the correct density in the appropriate flasks.