

Normal Human Dermal Fibroblasts-Neonatal (HDFn) Normal Human Dermal Fibroblasts-Adult (HDFa) Instruction Sheet

Safety and Use Statement

This product is for Research Use Only. This product is not approved for human or veterinary use or for use in *in vitro* diagnostics or clinical procedures.

Lifeline recommends storing cryopreserved vials in liquid nitrogen vapor phase. Handle cryopreserved vials with caution. Always wear eye protection and gloves when working with cell cultures. Aseptically vent any nitrogen from cryopreserved vials by carefully loosening the vial cap in a biosafety cabinet prior to thawing the vials in a water bath. If vials must be stored in liquid phase, the vials should be transferred to vapor phase storage or -80°C for up to 24 hours prior to being thawed.

Basic Aseptic Technique

Medium and LifeFactors® should only be used in an aseptic environment, a Class II biological safety cabinet with front access and filtered laminar airflow, or an equivalent device. Always wear gloves and eye protection when working with these materials. Wipe or spray medium bottle and LifeFactors with 70% ethanol or isopropanol before opening, especially around the area of the cap. Allow these surfaces to dry completely before opening the bottle or vials. Transfer of medium or LifeFactors should be done with disposable sterile pipettes. Do not mouth pipette! Take up the volume needed into the pipette, being careful not to touch the sterile tip to the rim of the container or any other surface. Close the container and open the container into which the transfer is being made, again being careful not to touch any surfaces with the sterile tip. Transfer the material and close the container. Wash your hands before and after working with cell cultures. Do not block airflow in a laminar flow hood as this may compromise sterility. Ensure that biological safety cabinets are certified routinely and that the HEPA filters are replaced regularly.

Cryopreserved Vials

Normal HDFn and HDFa are sold as cryopreserved vials and are shipped in insulated packages containing dry ice to ensure the cells remain in a cryopreserved state. To maintain the cells' integrity, unpack the products immediately upon receipt and store at a temperature lower than -150°C or in the vapor phase of a liquid nitrogen dewar. If the cells are to be thawed and plated within 24 hours, they may be stored at -80°C. Do not store the vial(s) for more than 24 hours at -80°C as the cells will slowly degrade at this temperature.

Medium Preparation

FibroLife® Basal Medium contains no growth factors, antimicrobials or phenol red. To support cell proliferation you must add FibroLife Serum-Free LifeFactors (LS-1010) or FibroLife S2 LifeFactors (LS-1038) to the basal medium (see FibroLife Medium instructions). Antimicrobials and phenol red are not required for cell proliferation, but may be purchased separately.

Pre-warming Medium

If using less than 100 mL of medium, Lifeline® recommends warming only the volume needed in a sterile conical tube. Repeated warming of the entire bottle over extended periods may cause degradation of the medium and reduced shelf life. When warming the entire bottle of medium, Lifeline recommends using a Lifeline water bath sleeve (included with medium) to help protect the medium from contaminants in the 37°C water bath. Medium will warm to 37°C in 10-20 minutes, depending on the volume. Do not leave medium in water bath for extended periods.

Thawing and Plating Cryopreserved Cells

Remove vial from dewar and check the cap to be sure that the vial is securely sealed. Spray the vial with 70% ethanol or isopropanol and transfer it to a biosafety cabinet. Allow it to dry thoroughly and carefully loosen the cap to vent any liquid nitrogen that may have entered the vial. Recap the vial and hold only the bottom half of the vial in a 37°C water bath for approximately one minute or until vial is almost completely thawed—a small amount of ice should still be visible. To avoid potential contamination, do not allow the vial cap to make contact with the water. Do not over thaw as this may damage the cells. Dry the vial, spray the exterior of the vial with 70% ethanol or isopropanol and place the vial in a biological safety cabinet and allow it to dry. Carefully remove the cap to avoid contamination or spatter. Gently resuspend the cells in the vial using a 1 or 2 mL sterile pipette. Do not centrifuge; the cells may be directly plated from the vial. Plate cells into pre-warmed, fully supplemented FibroLife Serum-Free Medium or FibroLife S2 Medium in desired culture vessel at a density of 2,500 cells per cm². Gently rock the culture vessel side-to-side and forward to backward to evenly distribute cells within the vessel. Place seeded culture vessel in the incubator at 37°C, 5% CO₂. Refeed the cells after they have attached. Refeeding should occur 4-36 hours after inoculation to remove cryopreservation reagents.

Standard Calculation for Plating of Cells

Gently re-suspend the cells evenly in the medium. Using a clean hemacytometer and aseptic technique, remove 20 µL of the cell suspension to a separate tube, such as a microcentrifuge tube. Add an equal volume of 0.4% Trypan Blue solution to the cell suspension in the microcentrifuge tube and allow it to sit for 1 to 5 minutes. Place 10 µL of the cell suspension into one chamber of the hemacytometer. Count a minimum of 4 quadrants on the hemacytometer (see diagram below). Dead and dying cells are permeable to Trypan Blue, viable cells will not be blue. For accurate cell counts, optimal number of cells per quadrant should be 25-75 cells. After counting the cells, calculate the average of the 4 quadrants. Take the cell count average and multiply by the dilution factor and by 10⁴ to get the number of cells per mL. Multiply the desired seeding density (2,500-5,000 viable cells per cm²) by the surface area of the vessel(s) to be inoculated. This will give you the total number of cells to inoculate one vessel. Divide the number of cells needed to inoculate one vessel by the total number of cells in the cell suspension. This will give you the volume of cell suspension with which to inoculate each vessel. Inoculate the cells into the culture vessels prepared with pre-warmed culture medium. Mix gently to evenly distribute the cells and place culture vessels into the incubator at 37°C, 5% CO₂.

Sample calculation:

Average viable cells per quadrant = 31

31 cells/quadrant x 10,000 quadrants/mL x 2 (dilution factor*) = 620,000 cells/mL

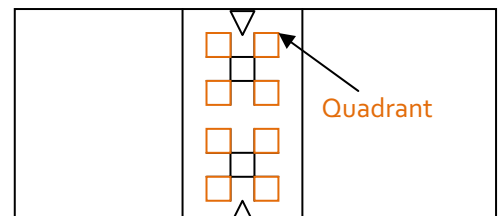
*if using equal volumes of Trypan Blue and cell suspension.

Inoculating a T-75 flask at 2500 cells/cm²:

2500 x 75 = 187,500 cells/flask

Calculate volume of cell suspension to inoculate each flask with:

187,500 cells/flask divided by 620,000 cells/mL = 0.302 mL/flask



Passaging Cells

Normal Human Dermal Fibroblasts may be passaged once the culture is 80-90% confluent and actively proliferating. Fibroblasts are not contact inhibited, however Lifeline® recommends that fibroblasts be passaged before reaching confluence since post-confluent cells exhibit slower proliferation after passaging. Lifeline recommends using the TrypKit™ Subculture Reagent Kit (LL-0013). Aspirate the medium from the culture vessel. Rinse the flasks with Lifeline's Phosphate Buffered Saline (PBS) by adding at least 1.0 mL of PBS (CM-0001) per each 5 cm² and gently tilting the flask to cover the surface with PBS. Aspirate the PBS from the culture vessel, repeat the rinse if desired. Add at least 0.5 mL of Lifeline's 0.05% Trypsin/0.02% EDTA (CM-0017) to the vessel for each 25 cm². Swirl gently to ensure all cells are coated with the Trypsin/EDTA. Observe the cells carefully under the microscope. When the cells round up they are ready to be released. This normally takes from 1-3 minutes depending on the confluence of the cells. Do not over trypsinize as this may damage the cells. Detach the cells by gently striking the culture vessel against the palm of your hand several times. Observe the cells under the microscope to be sure they have become detached. Once the cells are fully detached, add Lifeline's Trypsin Neutralizing Solution (CM-0018) using a volume equal to the amount of Trypsin/EDTA that was originally used. Gently swirl to ensure all of the trypsin solution is neutralized. Using aseptic laboratory techniques, pipette the cells into a sterile centrifuge tube. Collect the remaining cells by rinsing the culture vessel with at least 1.0 mL of PBS per each 5 cm² and pipetting the cells into the sterile centrifuge tube. Check culture vessel under the microscope for cells still attached and repeat steps if necessary to retrieve all the cells from the vessel. All steps must be completed under aseptic conditions in a biological safety cabinet. Centrifuge cells at 150 x g* for 3-5 minutes. For best results, calculate speed for individual centrifuge type. Time may also be centrifuge dependent. Do not over centrifuge cells as this will cause cell damage. After centrifugation, the cells should form a clean loose pellet. Please consult CellSystems® technical service department if issues arise from trypsinization or centrifugation. Aspirate neutralized trypsin from the centrifuge tube and re-suspend the cell pellet in pre-warmed FibroLife Medium by gently pipetting up and down with a 2 or 5 mL

*To calculate RCF ('x g')

$$RCF = 0.0001118 \times (rpm)^2 \times r$$

r = rotational radius in centimeters

rpm = rotations or revolutions per minute

Recommended Feeding Guidelines:

Guidelines for a T-25 Flask. Adjust volumes according to culture surface area.
Every other day, remove medium and feed with 5 mL of fresh supplemented medium.
Most cultures which are 50% confluent will be ready for passage the following day and should be fed with 7 to 8 mL of supplemented medium.
<u>Do not use more than 10 mL of medium per 25 cm² of culture surface to ensure that the media depth is at a level where gas diffusion will be sufficient to support the cells' requirements for oxygen.</u>

The depth of the medium affects gas diffusion gradients through the culture medium to the cells. The volumes of medium recommended in this table result in a range of depths between 2mm and 5mm, which is compatible with general recommendations, 10ml being at the maximum depth allowable (5mm).

Lifeline Technical Note: There are different and often contradictory terminologies used by cell culture companies to define the passage number of cells. Lifeline's designation of 'primary cells' are cells that have been isolated from tissue, plated onto culture vessels, expanded, harvested and cryopreserved. The term 'secondary' indicates that the cells have been isolated, plated and expanded in culture vessels twice before being harvested for cryopreservation.

CellSystems® Biotechnologie Vertrieb GmbH · Langeler Ring 5 · D 53842 Troisdorf · Germany

phone +49 (0)2241 25515 0 · fax +49 (0)2241 25515 30 · info@cellsystems.biz · www.cellsystems.biz

©2010 Lifeline Cell Technology. All Rights Reserved

Quick Steps for Cell Culture:

1. Always wash hands before and after working with cell cultures.
2. Always wear eye protection and gloves when working with cell cultures.
3. Store cryopreserved cells in liquid nitrogen upon receipt.
4. When working with cells or medium, always use a certified biological safety cabinet.
5. Handle cryopreserved vials with caution. Aseptically vent any nitrogen from cryopreserved vials in a biosafety cabinet prior to thawing in a water bath.
6. Feed cells using pre-warmed Lifeline® culture medium according to feeding chart.
7. When cells are 80-90% confluent and actively proliferating, passage cells using Lifeline subculture reagents.
8. Rinse cultures with PBS.
9. Trypsinize cells until rounded, do not over trypsinize.
10. Add TNS to stop trypsinization.
11. Add more PBS to rinse all the cells from the culture surface.
12. Centrifuge at 150 x g for 3-5 minutes. Adjust speed and time as appropriate for your centrifuge.
13. Aspirate solution from centrifuge tube, add pre-warmed Lifeline culture medium and gently re-suspend cells.
14. Count cells using a hemacytometer, re-plate at 2,500-5,000 per cm² in vessel containing pre-warmed culture medium.
15. Incubate cells using 1.0 mL of culture medium per 5 cm² at 37°C, 5% CO₂.
16. For any question on cell handling, please contact CellSystems® technical service. We are here to help.

Product	Catalog No.
Cryopreserved Normal Human Dermal Fibroblasts-neonatal (500,000 cells/vial)	FC-0001
Cryopreserved Normal Human Dermal Fibroblasts-adult (500,000 cells/vial)	FC-0024
FibroLife® Serum-Free Medium Complete Kit (FibroLife Basal Medium, FibroLife Serum-Free LifeFactors® Kit)	LL-0001
FibroLife® S2 Medium Complete Kit (FibroLife Basal Medium, FibroLife S2 LifeFactors® Kit)	LL-0011
TrypKit™ Subculture Reagent Kit	LL-0013

Notes:

CellSystems® Biotechnologie Vertrieb GmbH · Langelier Ring 5 · D 53842 Troisdorf · Germany
phone +49 (0)2241 25515 0 · fax +49 (0)2241 25515 30 · info@cellsystems.biz · www.cellsystems.biz
©2010 Lifeline Cell Technology. All Rights Reserved