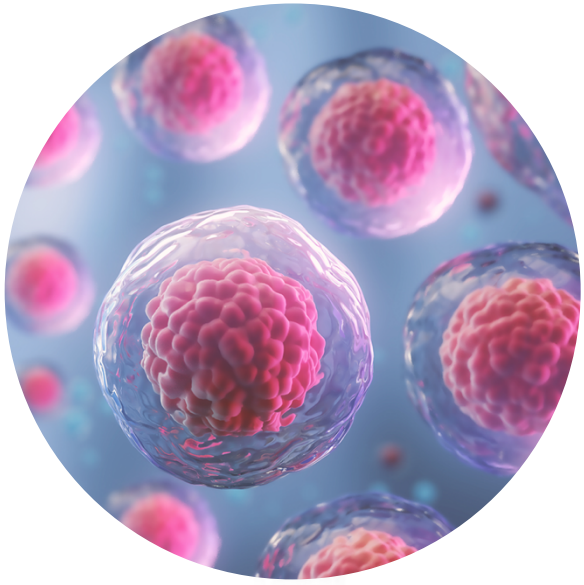
Instruction Sheet

Normal Human Dermal Fibroblasts-Neonatal, Xeno-Free § (HDFn-XF)

FC-0037
LM-0013

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.



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Safety and Use Statement

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.

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 vapor phase.
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 to 90% confluent and actively proliferating, passage cells using Lifeline Xeno-Free subculture reagents.
8. Rinse cultures with PBS.
9. Trypsinize cells until rounded, do not over trypsinize.
10. Add TNS XF to stop trypsinization.
11. Add more PBS to rinse all the cells from the culture surface.
12. Centrifuge at 150 x *g* for 3 to 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 to 5,000 cells per cm^2 (or use a split ratio of 1:5 to 1:20) in vessel containing pre-warmed Lifeline culture medium.
15. Incubate cells using 1 mL of culture medium per 5 cm^2 surface area at 37°C, 5% CO_2.
16. For any question on cell handling, please contact technical service. We are here to help.

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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.

§Lifeline documents that all materials used in the manufacture of products which are labeled 'Xeno-Free' have never come into contact with material of animal (non-human) origin and are not of animal (non-human) origin. FibroLife Xeno-Free Complete Medium contains human serum.

Product	Catalog Number(s)
HDFn-XF, Normal Human Dermal Fibroblasts-Neonatal, Xeno-Free§, Primary – 500,000 cells/vial	FC-0037
FibroLife™ Xeno-Free Complete Medium – 500 mL	LM-0013
TrypKit™ Xeno-Free Subculture Reagent Kit	LL-0043
FrostaLife™ Xeno-Free Cryopreservation Solution, 100 mL	LM-0019

Basic Aseptic Technique

Cells, and medium 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 all bottles and vials with 70% ethanol or isopropanol, especially around the area of the cap, before placing them in the biological safety cabinet. Allow these surfaces to dry completely before opening the bottle or vials. Transfer cells, medium or LifeFactors 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 Human Dermal Fibroblasts-Neonatal, Xeno-Free (HDFn-XF) are sold as cryopreserved vials and are shipped in biodegradable 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 for more than 24 hours at -80°C, as the cells will slowly degrade at this temperature.

Medium Preparation

FibroLife™ Xeno-Free Complete Medium (LM-0013) is fully supplemented with growth factors and shipped frozen. Thaw FibroLife Xeno-Free Complete Medium overnight at 2 to 8°C; 500 mL bottles may take two days to thaw. Alternatively, the medium can be placed in a 25 to 37°C water bath to thaw (see FibroLife Xeno-Free Complete Medium instructions). Multiple freeze/thaw cycles are not recommended.

A vial of Xeno-Free Penicillin/Streptomycin (XF-PS; LS-1073) is included in FibroLife Xeno-Free Complete Culture Kit (LL-0048) for your convenience. The use of XF-PS is recommended to inhibit potential bacterial contamination of eukaryotic cell cultures. Xeno-Free phenol red is not available at this time.

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 will cause degradation and reduced shelf life of the medium. Medium will warm to 37°C in 10 to 30 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 splatter. 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 FibroLife™ Xeno-Free§ Complete Medium in the desired culture vessel at a density of 2,500 to 5,000 cells per cm^2. Flasks with vented caps, commonly referred to as filter caps, are strongly recommended. Gently rock the culture vessel from side to side and front to back to evenly distribute cells within the vessel. Place seeded culture vessel in a 37°C, 5% CO_2 incubator. Refeed the cells after they have attached (approximately 4 to 36 hours after inoculation) to remove cryopreservation reagents.

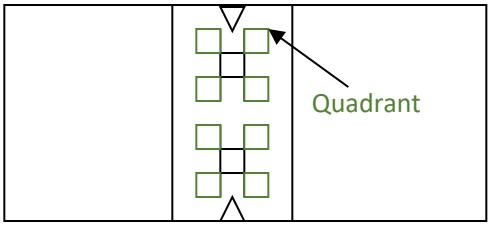
Standard Calculation for Plating of Cells

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. Perform cell counts between 1 to 5 minutes after the addition of Trypan Blue to the cell suspension. Place 10 µL of the cell suspension into at least 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 and will look completely blue. For accurate cell

counts, optimal number of cells per quadrant should be 25 to 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 10^4 to get the number of cells per mL. Multiply the desired seeding density (2,500 to 5,000 viable cells per cm^2) 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% CO_2.

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 2,500 cells/cm^2:
2,500 cells/cm^2 x 75 cm^2 = 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-Neonatal, Xeno-Free§ may be passaged once the culture is 80 to 90% confluent and actively proliferating. HDFn-XF 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™ Xeno-Free Subculture Reagent Kit (LL-0043). Aspirate the medium from the culture vessel. Rinse the flasks with Lifeline's Phosphate Buffered Saline (Xeno-Free) (PBS; CM-0001) by adding at least 1 mL of PBS per each 5 cm^2 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 2 mL of Lifeline's Trypsin/EDTA Xeno-Free (CM-0046) per each 25 cm^2 to the vessel. Swirl gently to ensure all cells are coated with the Trypsin/EDTA Xeno-Free. Observe the cells carefully under the microscope. When the cells round up they are ready to be released. This normally takes from 2 to 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 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 Xeno-Free (TNS XF; CM-0047) using a volume equal to the amount of Trypsin/EDTA Xeno-Free 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 mL of PBS per each 5 cm^2 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 the cells at 150 x g* for 3 to 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 Lifeline's technical service department if issues arise from trypsinization or centrifugation.

Aspirate the neutralized trypsin solution from the centrifuge tube and resuspend the cell pellet in pre-warmed FibroLife™ Xeno-Free Complete Medium by gently pipetting up and down with a 2- or 5-mL pipette. Count cells using a hemacytometer, and re-plate at 2,500 to 5,000 cells per cm^2 **(or use a split ratio of 1:5 to 1:20)** in vessel containing the respective pre-warmed culture medium.

***To calculate RCF (x g)**

RCF = 0.00001118 × $(rpm)^2$ × 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 FibroLife Xeno-Free Complete 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 FibroLife Xeno-Free Complete Medium.
Do not use more than 10 mL of medium per 25 cm^2 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.

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 2 mm and 5 mm, which is compatible with general recommendations, 10 mL being at the maximum depth allowable (5 mm).

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