Instruction Sheet

# FrostaLife™ and FrostaLife Xeno-Free§ Cryopreservation Solutions

LM-0015
LM-0019

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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## Quick Steps for Cryopreservation of Cells

*When culturing cells in a Xeno-Free§ environment, only use media and reagents that are documented to be Xeno-Free so as to maintain the Xeno-Free status.*

1. **Always wash hands before and after working with cell cultures.**
2. **Always wear eye protection and gloves when working with cell cultures.**
3. **Always use a certified biological safety cabinet when working with cells, medium, or reagents.**
4. **All steps must be completed under sterile conditions in a biological safety cabinet.**

5. Cells should be cryopreserved before cultures become fully confluent.
6. Thaw FrostaLife™ at 2 to 8°C overnight prior to use.
7. Prepare and label appropriate cryopreservation cryovial(s).

8. Remove cells from the culture vessel(s) using Lifeline's TrypKit™ or TrypKit Xeno-Free.
   a. Aspirate medium and rinse cultures with PBS.
   b. Trypsinize cells until rounded, do not over trypsinize—observe cells for detachment.
   c. Add TNS or TNS Xeno-Free to stop trypsinization.
   d. Add more PBS to rinse all the cells from the culture surface.
9. Centrifuge collected cell suspension at 150 x *g* for 3 to 5 minutes.
   a. Adjust speed and time as appropriate for your centrifuge.
   b. For best results, calculate speed for individual centrifuge type.
   c. Time may also be centrifuge dependent.
   d. Do not over centrifuge cells as this may cause cell damage.
10. After centrifugation, the cells should form a clean loose pellet.
11. Carefully aspirate neutralized trypsin from the centrifuge tube.
12. Gently resuspend cell pellet by adding a minimal volume (0.5 mL) room temperature cell culture medium or PBS.

    *Note: Volume of cell culture medium or PBS should be less than 10% of the final volume of FrostaLife being added in step 13.*
13. Perform a cell count and determine the volume of FrostaLife necessary to add to the cell suspension for cryopreservation to achieve a final cell density of $5 \times 10^5$ cells per mL to $2 \times 10^6$ cells per mL.
14. Add the correct volume of FrostaLife to the cells and mix gently.
15. Quickly dispense cell suspension into the cryovial(s).
16. Transfer the capped cryovial(s) to a Controlled Rate Cryopreservation Freezer or a Nalgene® Mr. Frosty® container in a -80°C freezer and initiate a controlled rate cryopreservation (i.e. -1°C per minute).

    *Note: Nalgene Mr. Frosty is a trademark of Thermo Fisher Scientific.*
17. Transfer the cryovial(s) to the vapor phase of a liquid nitrogen dewar within 12 to 24 hours of cryopreservation.

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## Safety and Use Statement

FrostaLife and FrostaLife Xeno-Free Cryopreservation Solutions contain DMSO.

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

FrostaLife Xeno-Free Cryopreservation Solution and Trypsin Neutralizing Solution Xeno-Free contain human serum and should be handled as potentially infectious material. While the human serum used in these products have been tested, no known test method can guarantee the absence of Hepatitis B virus, Hepatitis C virus, HIV-1, HIV-2 or other infectious agents. All products which contain or have contacted human blood based products should be handled as Biosafety Level 2 (www.CDC.gov) and according to the Bloodborne Pathogen Standard 29 CFR 1910.1030.

## FrostaLife and FrostaLife Xeno-Free Cryopreservation Solution Storage and Preparation

FrostaLife and FrostaLife Xeno-Free Cryopreservation Solutions should be stored at –20°C. Thaw the FrostaLife products overnight at 2 to 8°C. Once thawed, the FrostaLife products may be stored at 2 to 8°C for up to 2 months. If less than 20 mL are used, aliquoting is recommended to minimize freeze/thaw cycles.

| Product | Part No. | Volume | Storage |
|---|---|---|---|
| FrostaLife Cryopreservation Solution | LM-0015 | 100 mL | –20°C<br>Once thawed, store at 2 to 8°C for up to 2 months. |
| FrostaLife Xeno-Free Cryopreservation Solution | LM-0019 | 100 mL | –20°C<br>Once thawed, store at 2 to 8°C for up to 2 months. |

| Other Recommended Products | Part No. | Unit | Components | Storage |
|---|---|---|---|---|
| TrypKit™ Subculture Reagent Kit | LL-0013 | Kit | PBS<br>0.05% Trypsin/0.02% EDTA<br>Trypsin Neutralizing Solution | RT<br>–20°C<br>–20°C |
| TrypKit Xeno-Free Subculture Reagent Kit | LL-0043 | Kit | PBS XF<br>Trypsin/EDTA XF<br>Trypsin Neutralizing Solution XF | RT<br>–20°C<br>–20°C |

*§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. FrostaLife Xeno-Free Cryopreservation Solution and Trypsin Neutralizing Solution Xeno-Free contain human serum.*

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## Basic Aseptic Technique

Reagents, cells, 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 all bottles and cryovials 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 cryovials. Transfer materials 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.

## Preparing Cells for Cryopreservation

Label the cryovial(s) prior to working with the cells. Most cells may be cryopreserved once the culture is approximately 80% confluent and actively proliferating. Lifeline® recommends that most cells be cryopreserved before reaching confluence, since post-confluent cells may not recover from the cryopreservation process.

Lifeline recommends using the TrypKit™ (LL-0013) or TrypKit Xeno-Free§ (LL-0043) Subculture Reagent Kit to remove cells from culture vessels. Aspirate the medium from the culture vessel. Rinse the flasks with Lifeline's Phosphate Buffered Saline (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 0.05% Trypsin/0.02% EDTA (T/E, CM-0017; or T/E Xeno-Free, CM-0046) per each 25 $cm^2$ to the vessel. Swirl gently to ensure all cells are coated with the T/E or T/E 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 (TNS, CM-0018; or TNS Xeno-Free, CM-0047) using a volume equal to the amount of T/E or T/E Xeno-Free that was originally used. Gently swirl to ensure all of the T/E or T/E Xeno-Free 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.

***To calculate RCF (x *g*):** **$RCF = 0.00001118 \times (rpm)^2 \times r$**
r = rotational radius in centimeters
rpm = rotations or revolutions per minute

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Aspirate the neutralized T/E or T/E Xeno-Free solution from the centrifuge tube and resuspend the cell pellet in a minimal amount (for example, 0.5 mL) of room temperature complete culture medium or PBS by gently pipetting up and down with a 2 or 5 mL pipette. Perform a cell count.

Determine the volume of FrostaLife™ or FrostaLife Xeno-Free needed based on the cell count and resuspend the cells for cryopreservation to a density of $5 \times 10^5$ cells per mL to $2 \times 10^6$ cells per mL. Volume of cells in room temperature complete culture medium or PBS (above) should be less than 10% of the total volume of FrostaLife. Once the cells have been resuspended in FrostaLife they must be dispensed into cryovial(s) and the freezing process must be started within 15 to 30 minutes depending on the number of cryovial(s) to be cryopreserved. Do not over-tighten or under-tighten the cap(s) as this may result in an unsafe condition once the cryovial(s) are removed from the liquid nitrogen vapor stage storage container. See page 3 for details.

## Standard Calculation for Counting Cells for Cryopreservation

Gently re-suspend the cells evenly. Using a clean hemacytometer and sterile technique, remove 20 µL of the cell suspension to a separate tube, such as a microcentrifuge tube. For a [1:10] dilution factor, add at least 180 µL of 0.4% Trypan Blue solution to the cell suspension in the microcentrifuge tube and allow it to sit for up to 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 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 number of viable cells per mL by the volume (mL) of the cell suspension from which the sample was taken. This will give you the total number of viable cells to be cryopreserved. Divide the total number of viable cells by the desired cryopreservation density ($5 \times 10^5$ to $2 \times 10^6$ cells per mL). Subtract the volume in which the cells were resuspended for the cell count. This will give you the volume of FrostaLife™ or FrostaLife Xeno-Free[§] to add to the cell suspension.

**Sample calculation:**

Average viable cells per quadrant = 31

62 cells/quadrant x 10,000 quadrants/mL x 10 (dilution factor*) = 6,200,000 cells/mL

*if using a [1:10] dilution factor, as described in the above paragraph.

**Total Number of Viable Cells to be Cryopreserved**

6,200,000 cells/mL x 0.5 mL cell suspension = 3,100,000 viable cells

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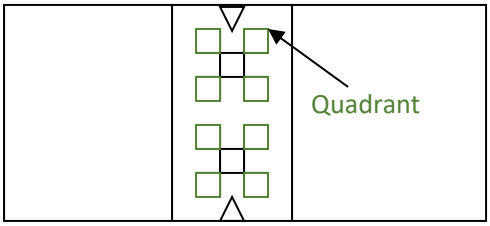
**Total Volume of Final Cell Suspension at Desired Cryopreservation Density of 500,000 cells/mL**

3,100,000 viable cells divided by 500,000 cells/mL = 6.2 mL total volume

**Total Volume of FrostaLife to Add to Cell Suspension**

6.2 mL total volume – 0.5 mL cell suspension = 5.7 mL FrostaLife or FrostaLife Xeno-Free




## Cryopreservation Methods

Various methods exist for the cryopreservation of cells. Controlled Rate Cryopreservation Freezers provide the best results in that they provide a controlled rate of cooling of the cell suspension at -1°C per minute. In the event that your laboratory does not possess a Controlled Rate Cryopreservation Freezer there are alternative methods and tools.

The Nalgene® Mr. Frosty® Cryo 1°C Freezing Container (catalogue number 5100-0001) is a common alternative to a Controlled Rate Cryopreservation Freezer. Please follow the instructions provided by the manufacturer. Essentially, cryovials are placed in a prepared Mr. Frosty Container, which is then placed in an ultra-low freezer (i.e. -80°C) for a minimum of 4 hours, but not longer than 24 hours.

Lifeline® recommends that cryovial(s) not be stored at -80°C for more than 24 hours as the cells' viability will slowly degrade at this temperature. **For long-term storage, cryovial(s) should be transferred to the vapor phase of a liquid nitrogen storage dewar or to a -150°C freezer.**

*(Nalgene Mr. Frosty is a trademark of Thermo Fisher Scientific)*

## The Lifeline Guarantee

Lifeline's rigorous quality control ensures sterility and performance to standardized testing criteria. Upon request, we will provide lot-specific QC test results, material safety data sheets, and certificates of analysis. See complete guarantee/warranty statement at lifelinecelltech.com or contact your technical representative for more information.

**For any questions on cell handling, please contact technical service at 877.845.7787. We are here to help.**