

KryoLife™ and KryoLife XF (Xeno-Free^S) Cryopreservation Solution 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. KryoLife contains DMSO.

Lifeline recommends using vials designed for cryopreservation and storing cryopreserved vials in liquid nitrogen vapor phase. Handle cryopreserved vials with caution. Always wear eye protection and gloves when working with cell cultures and vials. 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.

KryoLife XF contains human serum and should be handled as potentially infectious material. While the human serum used in this product has 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.

Medium Storage

KryoLife should be stored at -20°C. Thaw the KryoLife overnight at 2-8°C. Once thawed, KryoLife may be stored at 2-8°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
KryoLife Cryopreservation Solution	LM-0015	100 mL	-20°C
KryoLife XF Cryopreservation Solution	LM-0019	100 mL	-20°C

^SLifeline 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. KryoLife Xeno-Free Cryopreservation Solution contains human serum.

Basic Aseptic Technique

Reagents, 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 the 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.

Preparing Cells for Cryopreservation

Label the cryovials prior to working with the cells. Cells may be cryopreserved once the culture is ~80% confluent and actively proliferating. Lifeline® recommends that cells be cryopreserved before reaching confluence since post-confluent cells may not recover from the cryopreservation process. Lifeline recommends using the TrypKit™ Subculture Reagent Kit (LL-0013) or TrypKit Xeno-Free (LL-0043) to remove cells from culture vessels. Aspirate the medium from the culture vessel(s). Rinse the vessel(s) 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(s), repeat the rinse if desired. Add at least 1 mL of Lifeline's 0.05% Trypsin/0.02% EDTA (T/E, CM-0017; T/E Xeno-Free CM-0046) to the vessel(s) 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 or contract they are ready to be released. This normally takes from 1-3 minutes depending on the cell type and confluence. Do not over trypsinize as this may damage the cells. Detach the cells by gently striking the culture vessel(s) 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 (TNS, CM-0018; TNS Xeno-Free CM-0047) 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(s) with at least 1.0 mL of PBS per each 5 cm² and pipetting the cells into the sterile centrifuge tube. Check culture vessel(s) under the microscope for cells still attached and repeat steps if necessary to retrieve all the cells from the vessel(s). 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 Lifeline's technical service department if issues arise from trypsinization or centrifugation. Aspirate neutralized trypsin from the centrifuge tube and re-suspend the cell pellet in a minimal amount (for example, 0.5 mL) of room temperature PBS or complete culture medium by gently pipetting up and down with a 2 or 5 mL pipette. Perform a cell count.

Determine the volume of KryoLife™ or KryoLife XF needed based on the cell count and resuspend the cells for cryopreservation to a density of 5 x 10⁵ cells/mL to 2 x 10⁶ cells/mL. Volume of cells in PBS (above) should be less than 10% of the total volume of KryoLife. Once the cells have been resuspended in KryoLife they must be dispensed into vials and the freezing process must be started within 15 to 30 minutes depending on the number of vials to be cryopreserved. Do not over-tighten the caps as this may result in an unsafe condition once the vials are removed from the liquid nitrogen storage container. See page 3 for details.

*To calculate RCF ('x g')

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

r = rotational radius in centimeters

rpm = rotations or revolutions per minute

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. Add at least 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 number of viable cells per mL by the volume of the cell suspension (mL) 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 x 10⁵ to 2 x 10⁶ cells/mL). Subtract the volume in which the cells were resuspended for the cell count. This will give you the volume of KryoLife to add to the cell suspension.

Sample calculation:

Average viable cells per quadrant = 70

$$70 \text{ cells/quadrant} \times 10,000 \text{ quadrants/mL} \times 2 \text{ (dilution factor*)} = 1,400,000 \text{ cells/mL}$$

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

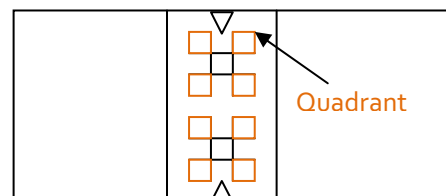
Determine the total number of viable cells harvested:

$$1,400,000 \text{ cells/mL} \times 4 \text{ mL (volume of cell suspension)} = 5.6 \times 10^6 \text{ viable cells}$$

Calculate volume of KryoLife needed to cryopreserve the

cells at 6 x 10⁵ cells/mL, subtract the resuspension volume:

$$5.6 \times 10^6 \text{ viable cells divided by } 600,000 \text{ cells/mL} = 9.33 \text{ mL} - 0.5 \text{ mL} = 8.83 \text{ mL}$$



Cryopreservation Methods

Various methods exist for the cryopreservation of cells. Controlled rate freezers allow researchers to fine tune the cryopreservation process to yield the best results. In the event that your laboratory does not possess a controlled rate cryopreservation freezer there are other, less expensive options.

One intermediate option is a Mr. Frosty® Freezing Container offered by Nalgene® Labware (5100-0001). The vials containing the cells are placed into the container. The container is then stored overnight in an ultra-low freezer (-70°C to -80°C). Within 18 to 72 hours the cells should be transferred to the vapor phase of a liquid nitrogen storage dewar or to a -150°C freezer.

Another 'low tech' option is to place the vials into a rack or box that will hold them upright. Place the rack or box into a Styrofoam box with a lid and place the box into an ultra-low freezer (-70°C to -80°C). Within 18 to 72 hours the cells should be transferred to the vapor phase of a liquid nitrogen storage dewar or to a -150°C freezer.

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 in order to maintain the xeno-free status of certain cell cultures.

1. Always wash hands before and after working with cell cultures.
2. Always wear eye protection and gloves when working with cell cultures.
3. When working with cells or medium, always use a certified biological safety cabinet.
4. Cells should be cryopreserved before cultures become fully confluent.
5. Thaw KryoLife™ at 2-8°C overnight prior to use.
6. Prepare and label appropriate cryopreservation vials.
7. Remove cells from the culture vessels using Lifeline's TrypKit™.
 - a. Aspirate medium and rinse cultures with PBS.
 - b. Trypsinize cells until rounded, do not over trypsinize—observe cells for detachment.
 - c. Add TNS to stop trypsinization.
 - d. Add more PBS to rinse all the cells from the culture surface.
 - e. Centrifuge at 150 x g for 3-5 minutes.
 - i. Adjust speed and time as appropriate for your centrifuge.
 - ii. For best results, calculate speed for individual centrifuge type.
 - iii. Time may also be centrifuge dependent.
 - iv. Do not over centrifuge cells as this may cause cell damage.
8. After centrifugation, the cells should form a clean loose pellet.
9. Carefully aspirate neutralized trypsin from the centrifuge tube.
10. Aspirate solution from centrifuge tube, add a minimal volume (0.5 mL) pre-warmed culture medium or PBS and gently re-suspend cells. (Volume of PBS or medium should be less than 10% of the final volume of KryoLife being added in step 12.)
11. Perform a cell count and determine the volume of KryoLife necessary to add to the cell suspension for cryopreservation to achieve a cell density of between 5×10^5 cells/mL and 2×10^6 cells/mL.
12. Add the correct volume of KryoLife to the cells and mix gently.
13. Quickly dispense cell suspension into the vials.
14. Transfer the capped vials to a specialized freezing container (e.g. Mr. Frosty®), or a rack or box inside a Styrofoam box and place the vials at -70°C to -80°C overnight. Alternatively, a controlled-rate cryopreservation freezer can also be used.
15. Transfer the vials to the vapor phase of a liquid nitrogen dewar within 18 to 72 hours of cryopreservation.
16. For any questions on cell handling, please contact technical service at 877.845.7787. We are here to help.

The Lifeline® Guarantee

Lifeline's rigorous quality control ensures sterility and performance to standardized testing criteria. If Lifeline's products do not meet our posted performance and quality standards, we will replace them at no charge or provide a full refund. Upon request, CellSystems® will provide lot-specific QC test results, material safety data sheets and certificates of analysis. See complete guarantee/warranty statement at cellsystems.biz or contact your CellSystems® technical representative for more information.

Product	Catalog Number(s)
KryoLife™ Cryopreservation Solution	LM-0015
TrypKit™ Subculture Reagent Kit	LL-0013
KryoLife XF Cryopreservation Solution	LM-0019
TrypKit Xeno-Free Subculture Reagent Kit	LL-0043

Notes:

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