PromoCell®

Cell cryopreservation in Cryo-SFM Plus

Application note

This application describes a detailed protocol for freezing and thawing cells using our advanced animal component-free and protein-free Cryo-SFM Plus cryopreservation medium.

Cryopreservation of cells is a fundamental and irreplaceable technique in cell culture, relevant to researchers across various fields. It allows for the long-term storage of cells, maintaining their integrity and viability over extended periods of time [1, 2]. This is particularly crucial for sensitive cell types, such as primary cells, stem cells, and immune system cells, whose viability can be compromised by cell freezing and thawing [3–5].

Cells are typically preserved in liquid nitrogen or its vapor phase at temperatures ranging from –150°C to –196°C [6]. This allows for cell expansion and banking for future use. However, frozen cells cannot be stored at temperatures above –125°C, the so-called glass-transition temperature, for a long time without experiencing progressive cell damage and loss of function [7, 8].

The main challenge with cryopreservation lies in ensuring that cells survive the freezing and thawing process, which involves profound changes in temperature (typically from 37°C to –196°C and then back to 37°C) [9]. The use of high-performance cryopreservation reagents is key to ensuring minimal cell loss and high cell viability and function after thawing [9, 10].

Background

Cryopreservation is an indispensable aspect of cell culture. However, it presents several challenges that can affect cell viability and functionality. Cryopreservation-induced stress during freezing and thawing can cause severe oxidative injury, cell shrinkage, and disruption of cellular structures, leading to cell damage and functional abnormalities [11, 12].

When performed too quickly, cell freezing may result in the formation of intracellular ice crystals, which can disrupt cell membranes and organelles [13]. On the other hand, slow freezing may result in the formation of ice crystals outside the cell, which may cause cell damage by increasing solute concentration around the cells [13, 14].

Dimethyl sulfoxide (DMSO) is a widely used cryoprotectant that is typically combined with other agents, such as glycerol, ethylene glycol, or sucrose [12]. Furthermore, serum and protein solutions are often used for cell freezing as the presence of proteins can reduce frost damage [15, 16]. However, high concentrations of cryoprotectants or their prolonged exposure to cells at higher temperatures are cytotoxic [17]. State-of-the-art cell freezing media should be animal component-free, protein-free, and non-cytotoxic, providing efficient cell protection even under suboptimal conditions.

Different cell types respond differently to cryopreservation [12], and it is particularly challenging to preserve their full biological potential and proliferation capacity.

Mesenchymal stem cells (MSCs) used in tissue engineering research are one prominent example [3]. Furthermore, cells may exhibit loss of membrane and organelle integrity after thawing, which can lead to alterations in enzymatic activity, cell signaling, and metabolic processes [18]. Such functional alterations in cells after thawing limit the reliability of experimental findings when cells are used in functional assays.

Several factors influence the extent to which cryopreservation affects cell viability and function. The freezing and thawing protocols are critical for successful cryopreservation [12, 19]. Controlled freezing with gradual temperature changes helps minimize cell damage [19]. Similarly, the thawing rate can significantly affect post-thaw cell integrity [4, 12]. The density and quality of frozen cells also play a role in the success of cryopreservation [10, 12].

The use of an effective cryopreservation medium is essential for minimizing functional alterations and loss of viability during cell freezing and thawing [12]. However, the type, concentration, and composition of the cryoprotectant can influence the effects of cryopreservation on cell viability and function [20].

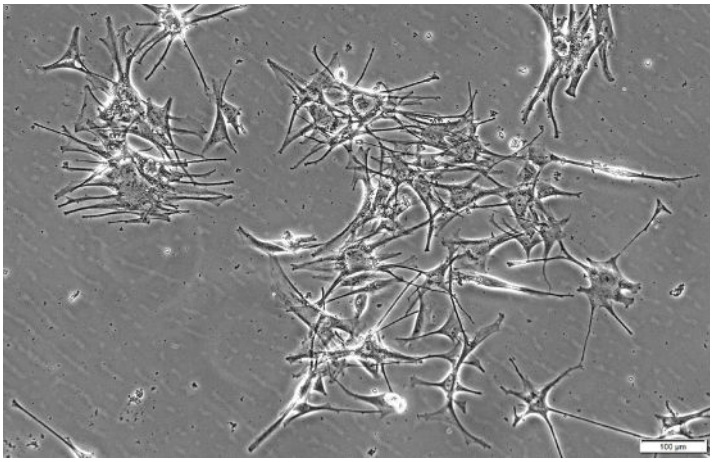
An ideal cryopreservation medium should be free of animal components to:

- reduce the risk of cross-species contamination and ethical concerns related to the use of animal products in research,
- effectively preserve the integrity and functionality of cells during the freezing and thawing process,
- align with regulatory requirements,
- have well-defined formulation to ensure batch-to-batch consistency and reproducibility [1, 2, 10].

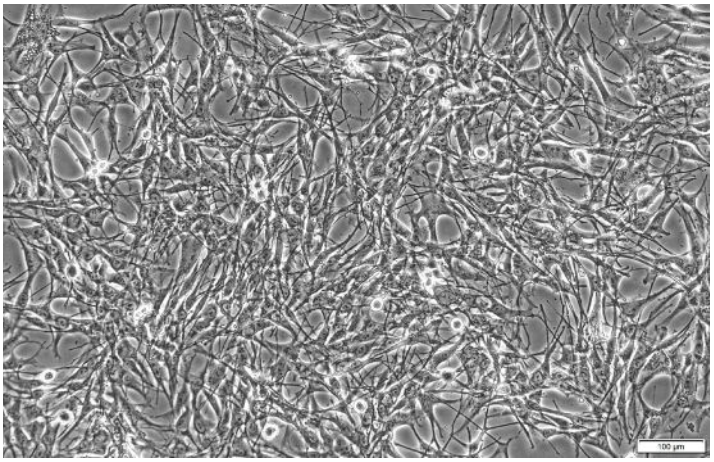
Moreover, cryopreservation formulations should be compatible with serum-free cell culture systems to allow maximum flexibility in downstream applications without interference from protein components [2, 12].

By addressing these aspects, Cryo-SFM Plus provides an optimized, well-defined, protein-free, and animal component-free solution combined with an antioxidant formulation for the cryopreservation of a wide range of cell types. The patented* formulation offers excellent results with various types of cells, including primary human cells, stem cells, and established cell lines. With Cryo-SFM Plus, even highly sensitive cells, like human primary melanocytes (Fig. 1), exhibit superior post-thaw cell health and integrity, enabling enhanced attachment and growth as compared to cells frozen in conventional freezing medium.

A

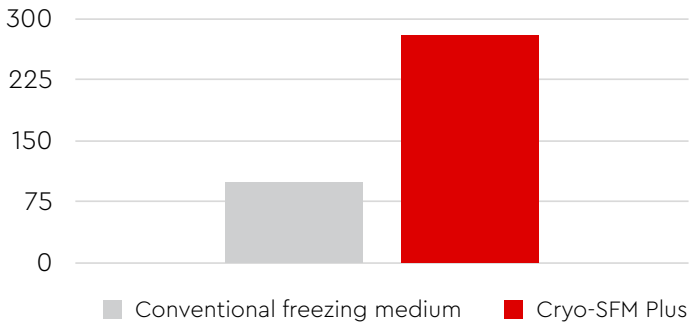


Conventional freezing medium



Cryo-SFM Plus

B Relative increase in cell number [%]



C Relative population doubling time [%]

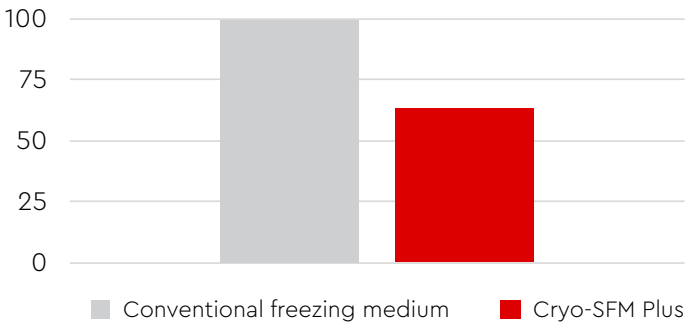


Fig. 1: Post-thaw quantitative proliferation analysis of melanocytes cryopreserved in different cell freezing media. (A) *Adult Normal Human Epidermal Melanocytes M3 (NHEM M3, C-12413) cryopreserved in conventional cell freezing medium (left) or Cryo-SFM Plus (right, C-29920/C-29922) were thawed and plated at 5000 cells per cm² on tissue culture treated plates in Melanocyte Growth Medium M2 (C-24300), and post-thaw cell proliferation was monitored. After thawing, NHEM M3 cryopreserved in Cryo-SFM Plus exhibited higher cell growth than the cells frozen in conventional cell freezing medium. Images were taken at the same time (magnification, 100x; scale bar, 100 µm).* ***(B)*** *Relative increase in cell number at the time of cell counting normalized to the number of cells thawed in conventional cell freezing medium. NHEM M3 frozen in Cryo-SFM Plus exhibited increased cell yield.* ***(C)*** *Post-thaw population doubling time normalized to the population doubling time of cells thawed in conventional cell freezing medium. Cells cryopreserved in Cryo-SFM Plus showed a 36% lower population doubling time (equivalent to 36% faster growth) than those cryopreserved in conventional cell freezing medium.*

**Patent issued in Germany and pending approval in other countries.*

I. Cryopreservation of cells

Materials

- Cells to be cryopreserved
- PromoCell Cryo-SFM Plus (C-29920, C-29922)
- Dulbecco's PBS without Ca^{2+}/Mg^{2+} (C-40232)
- Detachment reagent (for adherent cells)
- Complete culture medium
- Reagents for viable cell counting
- Cryovials or cryopreservation bags
- Controlled-rate freezer or isopropanol freezing container

Use aseptic techniques and a laminar flow bench.

1

Harvest the cells to be frozen

Note: Adherent cells, non-adherent cells growing in suspension, and large cell aggregates (e.g., intact tumorspheres or small tissue specimens) can be efficiently cryopreserved using Cryo-SFM Plus.

1a Detaching adherent cells

Aspirate the culture medium and wash the cell layer twice with a generous amount of PBS buffer without Ca^{2+}/Mg^{2+} (C-40232). Remove the buffer and add a suitable release agent, e.g., 100 µL/cm^2 Accutase (C-41310) or Trypsin/EDTA (C-41000).

Incubate the cells at 37°C for 2–10 minutes. The time required varies depending on the cell type and release agent used and should be limited to a necessary minimum. Monitor the detachment process microscopically. As soon as the cells begin to round off, release them completely by light tapping on the side of the cell culture container. Transfer the detached cells into a tube. Note that trypsin must be inactivated using Trypsin Neutralizing Solution (TNS, C-41100).

Dilute the cell suspension with an equal volume of complete culture medium and count the cells to determine the number of viable cells. Pellet the cells by centrifugation (e.g., for 3 minutes at 300 x g and room temperature) and continue with step 2.

1b Harvesting non-adherent suspension cells

Transfer the cells in suspension into a tube and count the cells to determine the concentration and total number of viable cells. After pelleting the cells by centrifugation (e.g., for 3 minutes at 300 x g and room temperature), continue with step 2.

2

Prepare the harvested cells for cryopreservation

Have Cryo-SFM Plus at hand (5°C – 25°C). Gently aspirate the supernatant covering the cell pellet, leaving 50–100 µL of the cell culture medium. Resuspend the cells in a suitable amount of Cryo-SFM Plus by carefully pipetting up and down using a serological pipette. The cell count per milliliter of the cell suspension should be between 100,000 and 100 million cells.

Rapidly distribute the cell suspension to suitable cell cryopreservation containers (e.g., cryovials or bags), transfer them immediately into a computer-controlled freezer, and start the cryopreservation process. Alternatively, the cells can be cryopreserved on dry ice for at least 4 hours in freezing containers filled with 2-propanol (e.g., Mr. Frosty).

Subsequently, remove the frozen cells from the device used for cryopreservation and transport them on dry ice at –80°C to the storage location without interruption of the cooling chain. Temporarily store the cells in liquid nitrogen or its vapor phase. Cells can be kept there indefinitely.

II. Thawing and seeding of cryopreserved cells

Materials

- Tissue culture vessels
- Complete culture medium
- Water bath (37°C)

Use aseptic techniques and a laminar flow bench.

1

Prepare the culture vessel

Calculate the required culture surface area according to the required plating density and fill a suitable cell culture vessel with a corresponding amount of culture medium (e.g., 0.2 to 0.3 mL/cm² of culture area). For optimal results, pre-equilibrate the culture vessel containing the medium in the incubator at 37°C and 5% CO_2 for at least 30 minutes before plating the cells.

2

Thaw and plate the cells

Note: The following section describes the process of thawing cells contained in 2 mL cryovials.

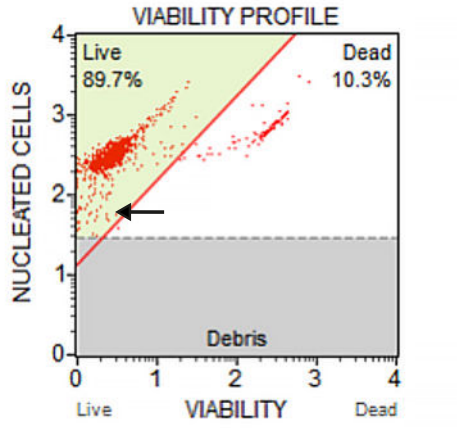
Prepare a tube containing at least nine times the volume of the cryopreserved cells with complete culture medium (room temperature), e.g., prepare 9 mL of medium for 1 mL of frozen cells. Remove the cryovial from the liquid nitrogen storage and immediately place it on dry ice, even for short transportation. Under a laminar flow bench, briefly twist the cap a quarter turn to relieve pressure, then retighten. Immerse the vial in a water bath (37°C) up to the height of the screw cap for 2 minutes (for 2 mL cryovials containing 1 mL of cells). Ensure that no water enters the thread of the screw cap.

Note: Thawing in the water bath should be limited to the minimal time required, i.e., until the content of the vial becomes liquid. A small residual ice clump is acceptable.

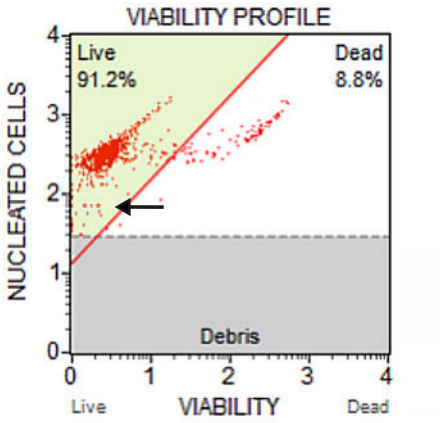
Remove the thawed cells from the water bath and thoroughly rinse the cryovial with 70% ethanol under a laminar flow bench. Then, aspirate the excess ethanol from the thread area of the screw cap. Open the vial and transfer the cells to a tube containing 9 mL of culture medium using a serological pipette.

Note: Optionally, the viable cell count can be determined to adjust to a particular seeding density (Fig. 2). It should be noted that post-thaw viability is of limited significance with respect to the assessment of cell health and/or performance of a freezing medium (compare Fig. 2 and Fig. 3).

Thereafter, pellet the cells by centrifugation (e.g., for 3 minutes at 300 x g and room temperature) and then aspirate the supernatant. Gently resuspend the cell pellet in a small volume of complete culture medium using a serological pipette. Seed the cells (taking into account the recommended seeding density for the respective cell type) into the culture vessel containing the pre-equilibrated complete medium. Place the cells in the incubator at 37°C and 5% CO_2 (Fig. 3).

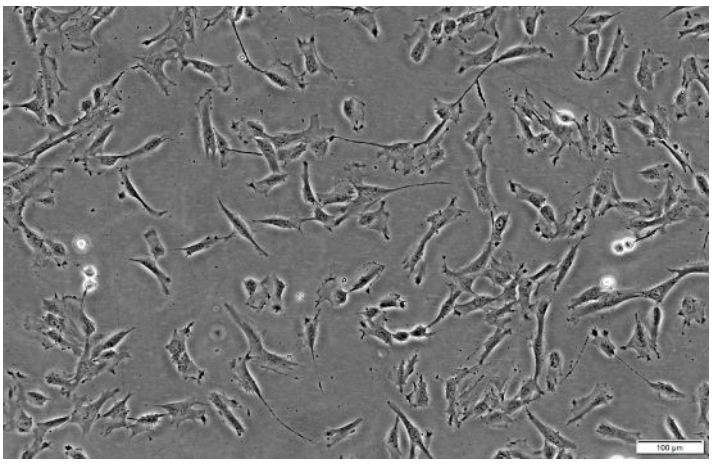


Leading competitor freezing medium

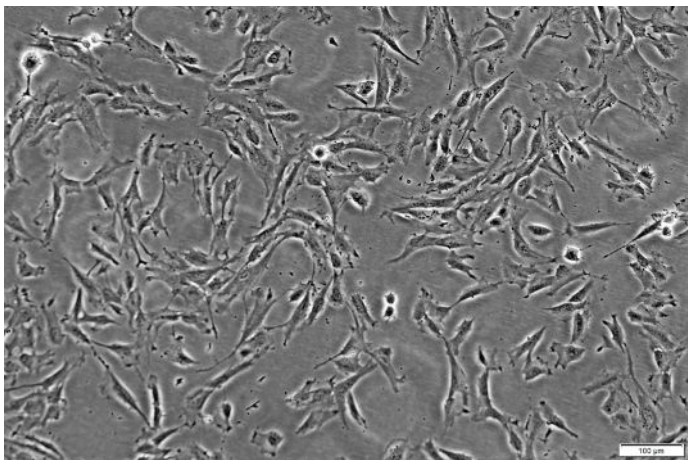


Cryo-SFM Plus

Fig. 2: Post-thaw cell analysis of mesenchymal stem cells from umbilical cord cryopreserved in different cell freezing media. *Human Mesenchymal Stem Cells (hMSC, C-12971) cryopreserved in the cell freezing medium of a leading competitor (**left**) or Cryo-SFM Plus (**right**, C-29920/C-29922) were thawed and subjected to post-thaw cell analysis. In addition to providing slightly higher cell viability, Cryo-SFM Plus protected the cells from damage during the freezing/thawing process, with higher efficiency than the competitor cell freezing medium. The arrows indicate fewer cells with impaired integrity when using Cryo-SFM Plus than when using a leading competitor's serum-free cell freezing medium.*



Leading competitor freezing medium



Cryo-SFM Plus

Fig. 3: Post-thaw proliferation of mesenchymal stem cells cryopreserved in different cell freezing media. *Human Mesenchymal Stem Cells (hMSC, C-12971) cryopreserved in competitor cell freezing medium or Cryo-SFM Plus (C-29920, C-29922) were thawed, counted, and plated at identical seeding densities. After 72 hours of cultivation in Mesenchymal Stem Cell Growth Medium 2 (C-28009), both cultures were imaged under a microscope. Note that the cells that had been frozen in PromoCell Cryo-SFM Plus (**right**) exhibited faster post-thaw growth and a higher cell yield (17% after 72 hours) than the cells thawed from the competitor cryopreservation medium (**left**). Microscopic assessment of hMSCs at 100x magnification; scale bar, 100 µm.*

Cryo-SFM Plus is a new cryopreservation solution that offers superior performance for the cryopreservation of various types of cells, including primary human cells, stem cells, cell lines, and cell aggregates (e.g., tumorspheres). Innovative antioxidants preserve the viability, attachment, and growth potential of cells after thawing, while the animal component-free and protein-free formulation eliminates the variability and interference caused by serum, proteins, or other undefined components. Cryo-SFM Plus is easy to use and complies with the highest technical and regulatory standards. This new cryopreservation medium provides a valuable tool for researchers who need a reliable and standardized method for storing and recovering their precious cell samples.

Related products

Product	Size	Catalog number
Cryo-SFM Plus	125 mL	C-29922
	30 mL	C-29920
Dulbecco's PBS, without Ca^{2+} / Mg^{2+}	500 mL	C-40232
Accutase-Solution	100 mL	C-41310
Trypsin/EDTA	30 mL	C-41000
	125 mL	C-41010
	250 mL	C-41020
Trypsin Neutralizing Solution	30 mL	C-41100
	125 mL	C-41110
	250 mL	C-41120
Human Mesenchymal Stem Cells (hMSC)	500,000 cells	C-12971
Normal Human Epidermal Melanocytes M3 (NHEM M3)	500,000 cells	C-12413
Melanocyte Growth Medium M2	500 mL	C-24300
Mesenchymal Stem Cell Growth Medium 2	500 mL	C-28009

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