Optimal Cryogenic Cooling

How to Develop a Process that Will Scale with Your Processing Volumes

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Introduction

Optimal Cryogenic Cooling

How To Develop a Process that Will Scale With Your Processing Volumes

The Goldilocks principle—stating that some things need to be "just right"—is incomplete. Exactly how is "just right" reached? This omission is particularly vexing in cryopreservation: one can be sure of the right temperature for cold storage while being uncertain over how, exactly, cooling should proceed.

It's essential to let science dispel such uncertainties. That's why we present evidence that reveals an optimal cooling profile—avoiding too fast, and too slow. Additionally, the evidence shows cooling rates should remain steady through most of the cooling period. That is, little or no advantage is conferred by rapid cooling steps.

Cryopreservation is about treading a sure path. It's crucial to give this step in the process meticulous care in order to support cell survival and function. Optimal cryogenic cooling is based on scientific research. Above all, cryopreservation is a science, not an art. And, in order to develop a process that will scale with your processing volumes, it's important to follow the data.

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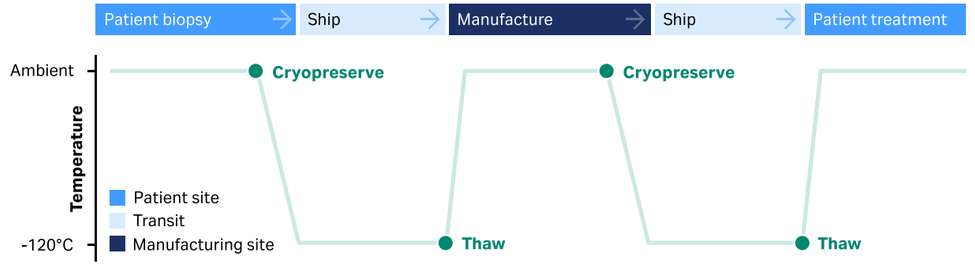
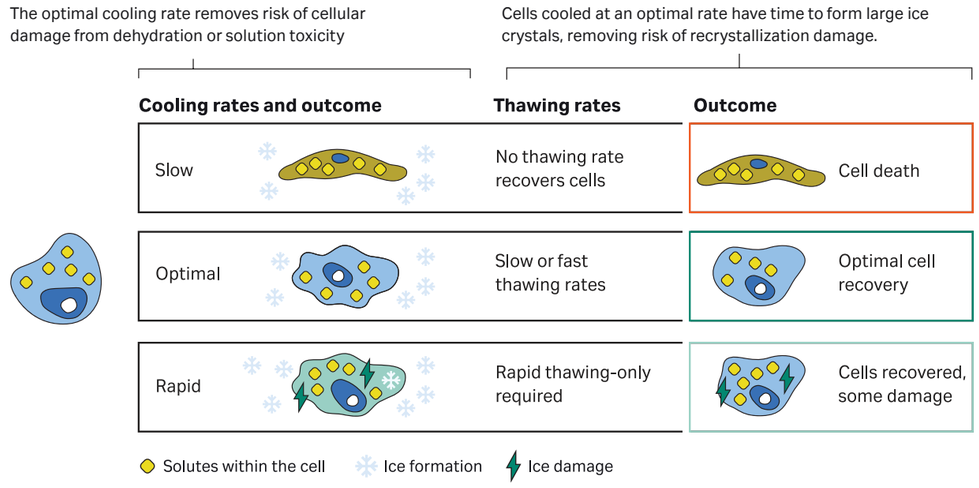
Cryochain elements of the cell therapy workflow and the importance of cooling and warming rates

The cryogenic cold chain steps within the cell therapy workflow



Slow, precisely controlled cooling, while essential for cell therapies, also allows for a wider acceptable range of thawing rates



Is a Rapid Cooling Step Needed?

dra_schwartz/Getty Images

Freezing Cells for Cryopreservation

Successful cell cryopreservation requires a controlled cooling rate. Cooling too rapidly or too slowly will lead to a poorer outcome. This is true because of the physical environment cells experience during cryopreservation. As ice forms in the extracellular solution, pure water is locked away as ice. As a result, cells are suspended in an increasingly concentrated solution, which dehydrates them. Cooling too slowly makes this solution toxic; cooling too rapidly prevents cells from dehydrating sufficiently, and intracellular ice forms.

Controlled-rate freezers, such as the electric-powered VIA Freeze™ series or liquid nitrogen (LN_2)-based freezers, are often programmed for linear cooling. A subset of researchers using LN_2-based freezers prefer to interrupt the linear cooling to include a "rapid-cooling nucleation step," which they believe is needed for successful cryopreservation. This step is hypothesized to improve ice nucleation – an important event for good post-thaw outcomes. A typical cooling profile for linear cooling with a rapid-cooling nucleation step is shown in Figure 1.

This study explores how a rapid-cooling nucleation step affects viable cell count and activity of four cell lines. Three conditions were tested: linear-rate freezing in a VIA Freeze™ system; linear-rate freezing in an LN_2-based freezer; and linear-rate freezing in an LN_2-based freezer, interrupted by a rapid-cooling step.

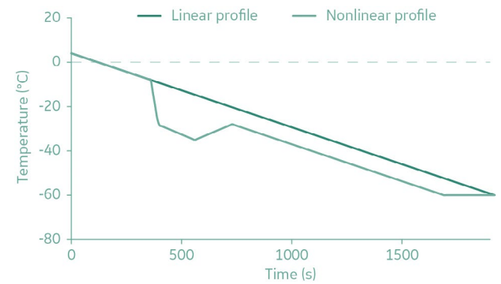


Fig 1. A linear 2°C/min cooling profile compared with a typical profile that includes a rapid-cooling nucleation step (1).

Table 1. Programmed parameters for experiment that includes rapid-cooling nucleation step.

Step	Cooling rate	Temperature range
1	-2°C/min	4°C to -8°C
2	-35°C/min	-8°C to -28°C
3	-2.5°C/min	-28°C to -35°C
4	+2.5°C/min	-35°C to -28°C
5	-2°C/min	-28°C to -60°C

Experimental Design

Cells in cryovials (1 mL fill volume) were cooled at 2°C/min from 4°C to -60°C in two controlled-rate freezers – VIA Freeze™ system (Cytiva) and a conventional LN_2-based freezer (Kryo 560, Planer). A third freezing method in this same LN_2-based freezer incorporated the rapid-cooling nucleation protocol from Table 1. Five cryovial samples, with 10% DMSO as cryoprotectant, were used for each condition. In all experiments, samples were plunged into LN_2 when the temperature reached -60°C. The cryovials were stored below -140°C for at least 24h, and then thawed. Thawed cells were placed into cell culture medium and allowed to grow in a standard humidified CO2 incubator at 37°C.

Table 1 lists the corresponding cooling rate sequence that was programmed into the LN_2-based controlled-rate freezer.

Four cell lines were examined: Chinese hamster ovary (CHO); HepG2 (human liver); MG-63 (human bone); and Jurkat (human suspension immune) cells. Viable cell count after thawing was determined using a Cytell Cell Imaging System. An alamarBlue™ bioassay was performed using a Tecan™ plate reader to evaluate metabolic function of cells after 3 h incubation. These

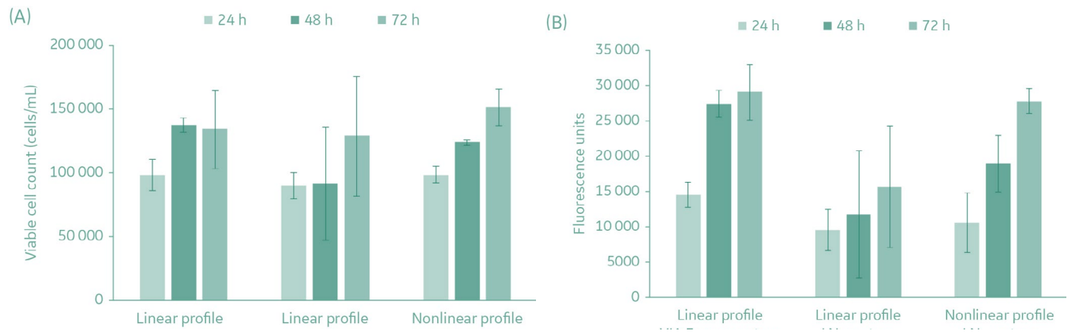


Fig 2. Post-thaw results for HepG2 cells. Cryovials were assessed 24, 48, and 72 h of culture post-thaw. (A) viable cell count, and (B) alamarBlue functionality. n = 5 ± SD.

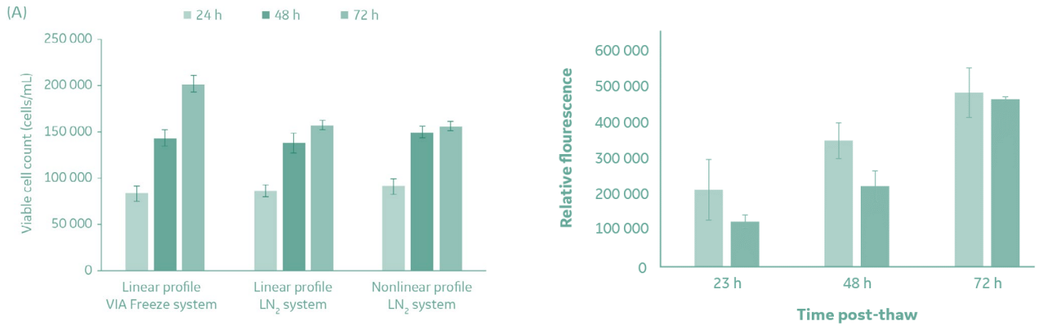


Fig 3. Post-thaw results for CHO cells. Cryovials were assessed 24, 48, and 72 h of culture post-thaw. (A) viable cell count, and (B) alamarBlue functionality. n = 5 ± SD.

functional tests were carried out at 24, 48, and 72 h of culture post-thaw.

Results From Different Freezing Methods

Figure 2 shows no significant difference in post-thaw results between HepG2 cells cooled in a LN_2-based freezer with or without a rapid-cooling nucleation step included. Furthermore, there is no significant difference in results using either freezer system and a linear cooling rate. The same pattern was observed with CHO cells (Fig 3), as well as Jurkat and MG-63 cells (data not shown, to keep the article brief).

Conclusions

In this study, including a rapid-cooling nucleation step in LN_2-based freezer did not improve post-thaw results for the four tested cell lines. No literature was found to support the hypothesis that a rapid-cooling step to induce ice nucleation is beneficial for cryopreservation.

Using Via Freeze™ System for Freezing Cells

In addition to demonstrating that a rapid-cooling step is unnecessary, the study shows comparable results using LN_2-based and VIA Freeze™ systems programmed with a linear cooling rate. Although cell viability and function are expected to be similar, the VIA Freeze™ system offers several advantages over LN_2-based controlled-rate freezers. Liquid nitrogen is not used for cooling, which avoids the associated contamination risks and supports GMP compatibility. Furthermore, using a VIA Freeze™ system is expected to reduce operating costs.

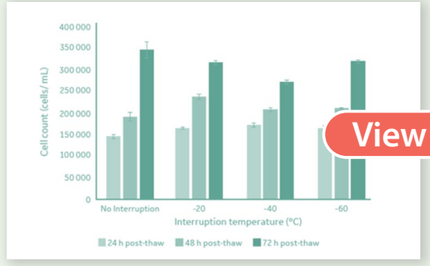
Reference

Diener B et al. A method for the cryopreservation of liver parenchymal cells for studies of xenobiotics. Cryobiology 30, 116–127 (1993).

Additional Resources...

Interrupted Cooling in Cryopreservation

Read this study to learn about the effect of power interruption during controlled cooling with a VIA Freeze™ system.



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Controlled Endpoint in Cryopreservation

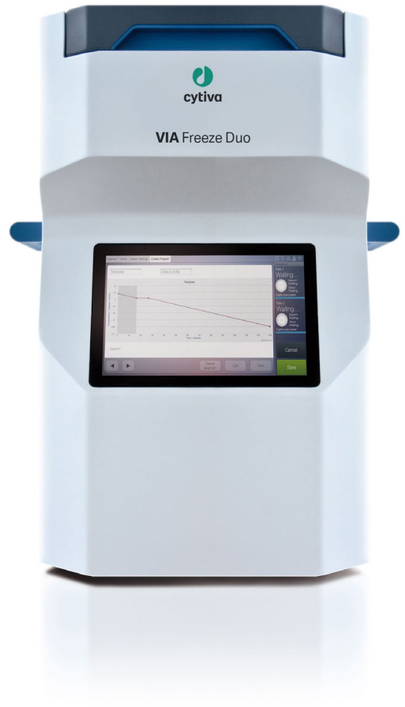


Fig 1. VIA Freeze™ controlled-rate freezer.

Cell Freezing by Cooling at a Controlled Rate

Successful cryopreservation of cells requires a controlled cooling rate. Cooling too rapidly or too slowly could lead to a poorer outcome.

A controlled cooling rate is needed because of the physical environment experienced by the cells during cryopreservation. As ice forms in the extracellular space, pure water is locked away as ice. This results in cells being suspended in an increasingly concentrated solution, which dehydrates cells. However, cooling too slowly makes this solution toxic. And cooling too rapidly prevents cells from dehydrating sufficiently; intracellular ice forms.

Controlled-rate cooling is achieved using equipment such as VIA Freeze™ controlled-rate freezers (Fig 1).

An important protocol parameter to understand is the temperature at which it is safe to stop the controlled cooling protocol and transfer cells to long-term storage, typically liquid nitrogen storage. Surprisingly, there have been few studies to define the endpoint at which cells can be transferred to storage conditions. Temperatures from -35°C to -130°C are in use.

This study explored the critical temperature for the endpoint of the controlled cooling phase. Defining that value would minimize the amount of time samples must be cooled in a controlled-rate freezer. By reducing cooling time per run, there is the potential to increase the number of runs per day.

Design Of Cooling Endpoint Study

To determine the biological implications of the controlled cooling endpoint temperature, cell samples in cryovials (1 mL fill volume) were cooled at 1°C/min in a VIA Freeze™ system over a range of temperatures from 4°C to -100°C (Fig 2).

At each selected temperature, sets of 5 samples were plunged into liquid nitrogen. The cryovials were stored below -140°C for at least 24 h, and then thawed.

Jurkat cells (human suspension immune) and Chinese hamster ovary (CHO) cells were evaluated. Dimethyl sulfoxide (DMSO, 10%) was used as cryoprotectant. After thawing, cells at each endpoint were grown in cell culture medium under standard conditions. Viable cell counts were determined after 24, 48, and 72 h of growth. A Cytell Cell Imaging System was used for analysis.

Results

According to Figure 3, temperatures at or below approximately -40°C are sufficient for CHO cell cryopreservation. Similar results were obtained with Jurkat cells (Fig 3), with a critical temperature between -40°C and -50°C. The same pattern was observed with HepG2 and MG-63 cell lines

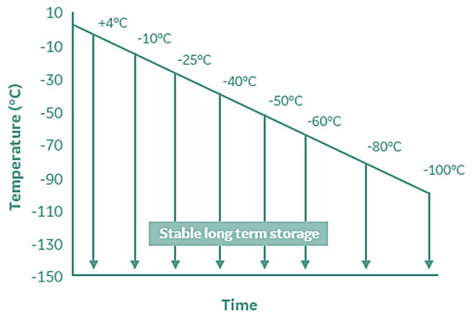


Fig 2. A schematic of the experimental design.

(data not shown, to keep the article brief).

Post-thaw viable cell counts of CHO and Jurkat cell samples at different cooling endpoints.
Post-thaw viable cell counts of CHO and Jurkat cell samples at different cooling endpoints.

Fig 3. Viable cell count for (A) CHO cells and (B) Jurkat cells at 24, 48, and 72 h of culture post-thaw with different endpoints during controlled-rate cooling. n = 5 ± SD.

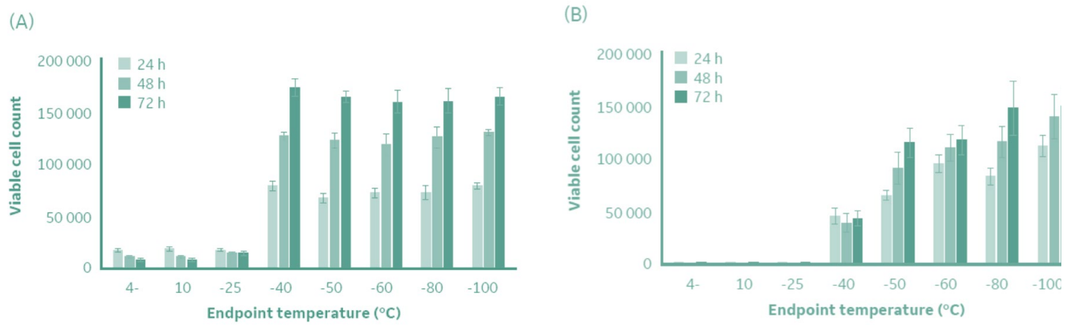


Fig 3. Post-thaw results for CHO cells. Cryovials were assessed 24, 48, and 72 h of culture post-thaw. (A) viable cell count, and (B) alamarBlue functionality. n = 5 ± SD.

Conclusions

This study demonstrates that the key temperature during linear cooling is between -40°C and -50°C, and further cooling offers no additional advantages. Physically, this finding can be attributed to an intracellular colloidal glass transition at -47°C. At this temperature the intracellular compartment solidifies, and the cells cannot respond osmotically. In addition, there is no free water to form intracellular ice. Thus, the key factors for cryopreservation-induced cell death are eliminated.

Advantages of Choosing a Higher Endpoint

Compared with cooling to -100°C, for example, a higher endpoint can be chosen. This means that cycle times can be shorter for cryopreservation protocols, allowing multiple runs in a day. A temperature such as -60°C is recommended to give a safety margin in case samples warm during transfer to long-term storage.

Because the maximum cooling rates VIA Freeze™ systems can achieve is temperature dependent, larger volumes can be processed when choosing -60°C as an endpoint over -80°C or -100°C.

Delivering LN_2-Free Large Volume Cryopreservation

A Proof-of-Concept Study with the VIA Freeze™ Quad Controlled-Rate Freezer

Fig 1. A view of the cryochamber within the VIA Freeze™ Quad unit with sample plates loaded with 8 × 70 mL cryobags held in metal cassettes.

Large-Volume Cryopreservation

The controlled cooling of small sample volumes is common in many scientific cryopreservation applications. The ability to cryopreserve larger volumes, for example in the development and production of many late-phase gene and cell therapies, is becoming increasingly desirable as more licensed treatments become available.

CAR T cell therapies, as well as blood products and cord blood, are some common examples of where large-volume cryopreservation is common. This is due to the large cell numbers required for effective treatment, as well as processing limitations. In all applications, it is critical that cell viability and function are maintained during the cryogenic processing and after thawing.

In many cases, liquid nitrogen (LN_2) is the default choice for cryopreservation, pumped into the cooling chamber directly. Use of LN_2 can give rise to concerns of contamination and sterility, safety, and additional running costs. Samples nearest to the LN_2 source will also cool at a different rate than those furthest away, leading to inconsistent cooling and concerns regarding cell viability.

This study demonstrates a cell freezing protocol that uses the LN_2-free VIA Freeze™ Quad controlled-rate freezer for the cryopreservation of up to eight cryobags and up to 560 mL total volume. Similar protocols can be applied to the VIA Freeze™ Uno and VIA Freeze™ Duo controlled-rate freezers for cryopreservation of up to 140 mL and 280 mL, respectively.

Using this VIA Freeze™ protocol, thermal profiles and biological outcome in terms of post-thaw viability are equal between different bags in a "rack" set-up and unaffected by the orientation of the cryobags (vertical or horizontal). Also, the same cooling rates and biological results are achieved whether cryopreserving only one bag, or eight, indicating that the proportion of loading capacity used does not impact VIA Freeze™ performance.

Methods for Freezing Multiple Cryobags

Two VIA Freeze™ multi-cryobag plates (Cytiva) were placed into a VIA Freeze™ Quad controlled rate freezer (Cytiva) with a VIA Freeze™ Quad 80 mm lid spacer (Cytiva). This set-up enables the cryopreservation of up to eight cryobags with a fill volume of 70 mL each (Fig 1).

Eight CryoMACS™ 250 bags (Miltenyi Biotech) were each filled with 70 mL of Jurkat cell suspension at a density of 3 × 106 cells/mL. The cryoprotective medium suspending the cells consisted of 5% DMSO in RPMI-1640 (Gibco), supplemented with 10% HyClone™ Calf Serum, U.S. origin (Cytiva).

The cells were cooled using the protocol shown in Table 1 before being transferred to ultra-low temperature storage at -140°C (transfer to long-term storage is possible once temperatures fall below -50°C). As nucleation is a stochastic event, a hold step is used during cooling to allow ice formation and temperature equilibration.

After a minimum of 24 h storage, samples were thawed rapidly in a VIA Thaw™ CB1000 unit (Cytiva) and recultured. Viable cell count, through

Table 1.

Cryopreservation step	Purpose
Room temperature to 4°C at 1°C/min	At 4°C, samples were loaded to the VIA Freeze™ system, to reduce DMSO toxicity
4°C to -35°C at 0.75°C/min	Initial cooling and freezing of sample
30 minute hold at -35°C	A hold to allow the whole sample to freeze after ice nucleation
-35°C to -60°C at 0.75°C/min	Final cooling step to transfer temperature, held at -60°C for 15 min before transfer

fluorescein diacetate (FDA) staining, and metabolic activity, through resazurin sodium salt reduction, was assessed at 24, 48, and 72 h post-thaw using a Cytell Cell Imaging System (Cytiva) and GENios FL plate reader (Tecan), respectively.

Thermal tests were carried out with cryobags containing 70 mL cell-free cryopreservation medium. In one test, a t-type thermocouple was attached externally to each of the eight cryobags, and data recorded during cooling, to assess inter-sample variability.

In a second test, the effects of placing a bag horizontally or vertically in a VIA Freeze™ system were assessed. One cryobag was placed in a horizontal plate on one half of a VIA Freeze™ Quad unit with a thermocouple attached (Fig 2A). On the other half, four 70 mL cryobags were placed in cassettes held between vertical plates in a rack set-up (Fig 2B) with four thermocouples attached to separate points of one bag (Fig 2C) to also assess intra-sample thermal variation.

Results

Consistency Between Samples Cryopreserved in Large Volumes

An evaluation of post-thaw viability and metabolic activity was necessary to demonstrate the validity of the set-up for freezing cells in large volumes and the consistency that can be achieved across multiple samples.

Thermal profiles indicated that all cryobags in this large volume set-up experience similar cooling profiles throughout cryopreservation (Fig 3).

In turn, cells from all bags showed consistent recovery, with no significant effects on post-thaw viability or metabolic activity (Fig 4).

Viable cell counts and metabolic activity recorded for each of the eight bags in the large volume cryopreservation study.

Consistency Between Vertical and Horizontal Cryopreservation

Conventionally, approaches for the cryopreservation of large sample volumes involve laying cryobags horizontally within a controlled-rate freezer. This limits the total volume that can be loaded in one cooling cycle. This study compared the physical and biological impact of cryopreserving bags horizontally and vertically. The vertical set-up enables cryopreservation of multiple cryobags simultaneously, making more efficient use of the cooling space available.

Figure 5 (on the following page) indicates that there is no significant difference in post-thaw

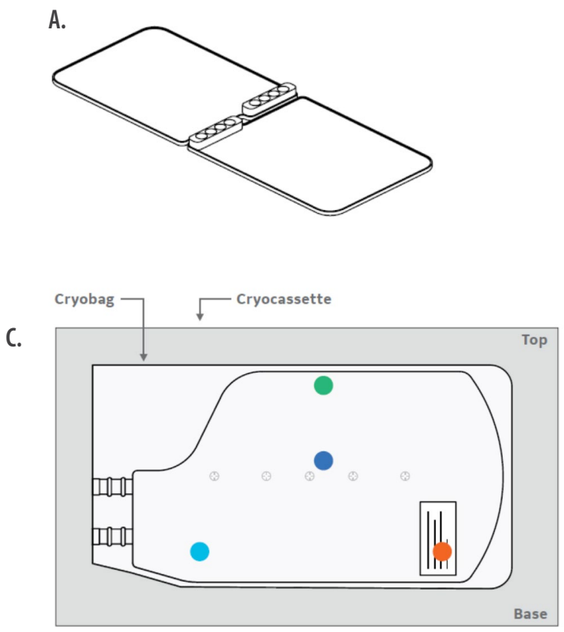


Fig 2. VIA Freeze™ cryobag plates for (A) horizontal and (B) vertical orientations. Vertical plate is adjustable to fit all cassette widths. (C) location and set-up of the cryobags during vertical freezing. Note that the larger volume of the bag is placed towards the base (i.e., sample plate) of the VIA Freeze™ sample area. Four thermocouples are attached to separate points on the cryobag (colored dots).

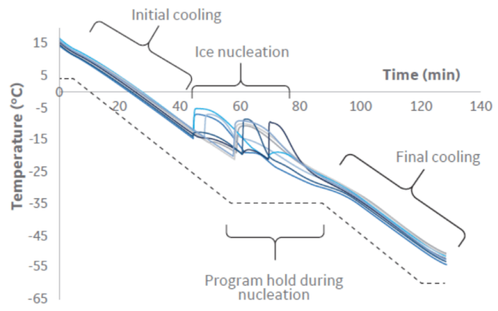


Fig 3. Cooling profile (dashed-black) programmed during the large volume cryopreservation, as well as the bag temperatures (blue) recorded in each of the eight cryobags filled with 70 mL volume each.

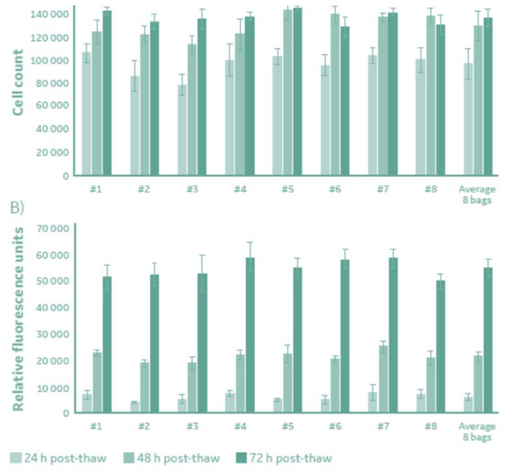


Fig 4. Analysis of samples from each of eight bags and average of bags. Two-tailed homoscedastic student's t-test shows no significant differences between the pairs ($P<0.01$). (A) viable cell count via fluorescein diacetate staining, and (B) metabolic activity read through resazurin sodium salt reduction. Cells were thawed then cultured under normal conditions for 24, 48, and 72 h. Data is n=5 technical replicates ± one standard deviation. Average of the eight bags (far right) is n=8 ± SD).

cell viability between samples cryopreserved in vertical and horizontal plates. There is also little difference in thermal profile between samples frozen horizontally and vertically, with multiple bags placed vertically having the same thermal profile as only one horizontal bag (Fig 6). In addition, Figure 6 demonstrates that there is minimal intra-sample thermal variation.

The VIA Freeze™ Quad system achieves these consistent thermal profiles by detecting the sample load automatically and applying an equal cooling profile, regardless of volume loaded. This approach makes sure that each bag is cooled consistently from the base, and is not insulated by other bags in the system as is often the case in liquid nitrogen systems. Enabling consistent cooling profiles between bags and between different cooling cycles, as demonstrated in this study, is essential for reliable recovery of cellular products.

Conclusions on Multi-Bag Cryopreservation Without Liquid Nitrogen

This study demonstrates that the VIA Freeze™ Quad controlled-rate freezer enables cryopreservation of large volumes (up to 560 mL) with high and consistent post-thaw recovery between all samples. The orientation of the cryobags during the freezing process (vertical or horizontal) made no significant difference to post-thaw cell viability.

The system provides consistent cooling profiles, rates, and biological outcome regardless of volume used, allowing cryopreservation of large volumes of precious biological samples in an LN_2-free process. This approach provides convenience and flexibility for making the most efficient use of the cooling space, with the added benefit of removing LN_2-related sterility and safety concerns.

The set-up and cooling program described can also be used with smaller volumes and other cryo-containers to suit user requirements.

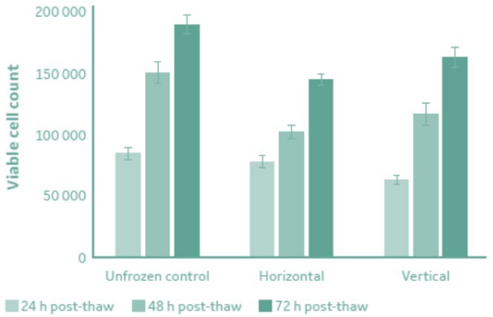


Fig 5. The viable cell count recorded for bags frozen in a low-volume horizontal set-up compared with a large volume vertical set-up, as well as unfrozen control. Cells were thawed then cultured normally for 24, 48, and 72 h. Two-tailed homoscedastic student's t-test shows no significant differences between the cryopreserved conditions ($P<0.01$). Data is n=5 technical replicates ± one standard deviation.

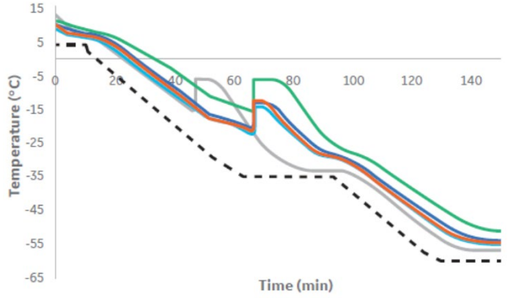
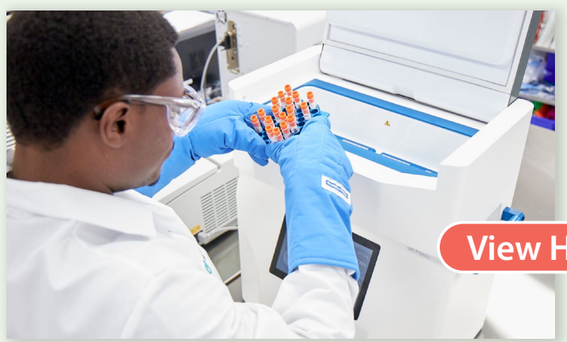


Fig 6. The thermal profiles observed when cryopreserving either horizontally (gray), or vertically (light blue, dark blue, orange and green, relating to thermocouple positions in Fig 2C), as well as the differences in thermal profile seen within a vertically cryopreserved bag. The programmed cooling profile is shown as black-dash.

Additional Resources...

Going Liquid Nitrogen-Free for Low-Impact Cryopreservation

This study demonstrates that the use of an LN2-free controlled-rate freezer will not only reduce financial costs for an organization, it will also reduce a cryopreservation center's carbon footprint.



Enabling LN_2-free Large-Volume Cryopreservation in Vials

A Proof-of-Concept Study with the Via Freeze™ Quad Controlled-Rate Freezer

microgen/Getty Images

Large-Volume Cryopreservation

Cryopreservation of small sample volumes is common in many scientific applications. However, there is increasing demand for large-volume cryopreservation, especially in cell and gene therapies, where treatments require large cell numbers.

Although cryobags are generally a good choice for the cryopreservation of large-volumes, cryovials provide a more robust alternative. They are also considerably easier to fill and empty, and are available in volumes up to 50 mL to meet high volume needs. However, the thermal performance of large-volume cryovials differs markedly from small vials and cryobags, which have wall thicknesses of typically 100 to 300 µm and so enable rapid heat transfer in and out of a biological sample.

By comparison, large-volume vials require thicker walls, typically up to 1 mm, to maintain structural security during cooling, thawing, storage, and transportation, and are made from polymers with poor thermal conductivity. This combination results in delayed heat transfer in and out of a sample, which can lead to thermal gradients within a large-volume sample.

The resulting cooling inconsistencies mean cells in the center of a vial can freeze more slowly than those nearer the edge, affecting their viability and function (1).

Such inconsistent cooling is also a consideration when freezing cells with liquid nitrogen (LN_2), often the default choice for cryopreservation. Samples nearest to where the liquid nitrogen is pumped into the cooling chamber cool at a different rate to samples further away. As well as potential sample variability, liquid nitrogen gives rise to concerns about contamination and sterility, safety, and high running costs.

This study demonstrates an optimized cooling protocol for cryopreserving large-volume cryovials in a VIA Freeze™ Quad controlled-rate freezer, avoiding the need for liquid nitrogen. Using a new sample plate, developed to accommodate large cryovials and increase the capacity of the VIA Freeze™ Quad freezer to 16 vials and a total of 480 mL, this protocol produces consistent cooling profiles. Samples are cooled evenly below the -60°C necessary for cryopreservation and show rapid recovery and post-thaw viability.

Methods for Cell Freezing Protocols and Analyses

Investigating Thermal Variation

Table 1 outlines an optimized cooling protocol for large-volume vials, refined to account for vial wall thickness, thermal conductivity of vial sample plates, and known biological constraints.

An initial temperature of -2°C is optimal for sample loading, as this is too warm for ice to form, but could prevent excessive DMSO toxicity.

The plastic walls of the vials result in a thermal lag between freezer and sample

Table 1. Cooling Protocol Optimized For Large-Volume Vial-Based Cryopreservation

Cryopreservation step	Purpose
Room temperature to -2°C at 1°C/min	The initial cooling to -2°C to minimize DMSO toxicity
10-minute hold at -2°C	A hold at -2°C to accomodate the thermal lag from thicker plastic cryovial, resulting in a liquid temperature just above 0°C, and allow sample loading
-2°C to -30°C at 1°C/min	Freezing of samples, reducing temperature to below their equilibrium freezing point where ice starts to form
30-minute hold at -30°C	A hold to allow the whole sample to freeze after ice nucleation
-30°C to -70°C at 0.75°C/min	Cooling resumes when samples are macroscopically frozen until temperature falls linearly to -70°C
10-minute hold at -70°C	A final hold enabling the vials to equilibrate before safe transfer to LN_2 at this temperature

temperatures. This is expected to be less than 10°C during the cooling stages and is accommodated by a 10-minute hold so as not to substantially affect overall cooling rates.

The hold at -30°C allows for any thermal deviation during ice nucleation to settle. Finally, the 10-minute hold at the end of the second cooling step makes sure all samples are below -60°C. As the intra-cellular glass transition is known to occur above -50°C, -60°C represents an acceptable temperature for transfer into long-term storage.

Two thermal tests demonstrated how this optimized cooling protocol accounts for the potential effect of thermal variation using large-volume cryovials.

Sixteen 50 mL AT-Closed Vial cryovials (Aseptic Technologies) containing 30 mL culture medium and 5% (v/v) DMSO were placed into four multi-vial sample plates (Cytiva) (Fig 1) and positioned in a VIA Freeze™ Quad controlled-rate freezer (Cytiva).

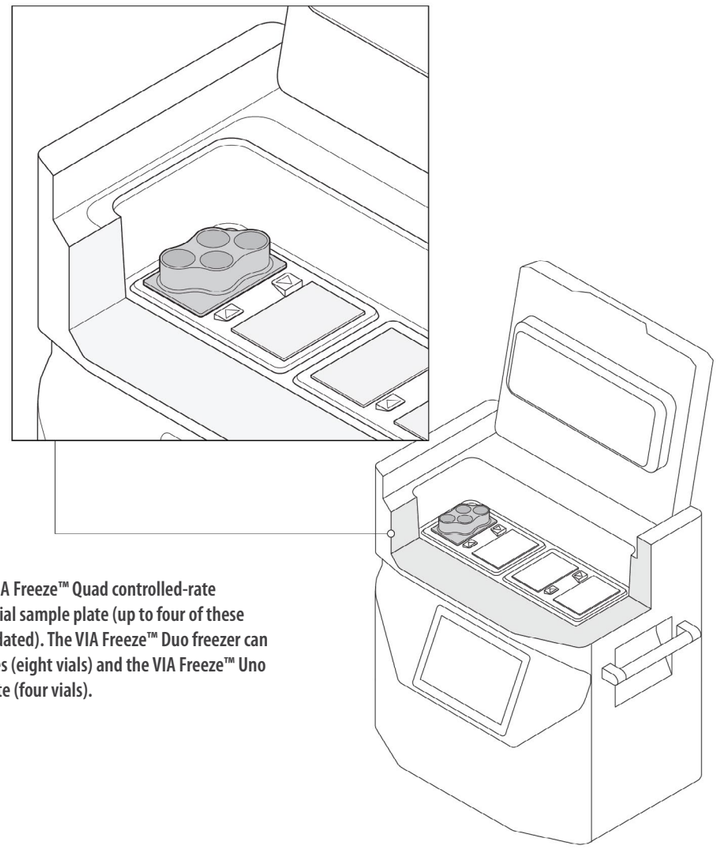


Fig 1. A cutaway image of the VIA Freeze™ Quad controlled-rate freezer loaded with one multi-vial sample plate (up to four of these sample plates can be accommodated). The VIA Freeze™ Duo freezer can accommodate two sample plates (eight vials) and the VIA Freeze™ Uno has capacity for one sample plate (four vials).

For the first thermal test, t-type thermocouples were randomly placed within the medium of eight vials to measure any inter-sample variability and the impact of thermal lag through the cryovial walls.

In the second thermal test, thermocouples were placed in two of 16 new cryovials in the VIA Freeze™ Quad freezer to measure intra-sample variability when cooling large 30 mL volumes. One thermocouple was placed near the base of each vial, two towards the middle, and a fourth near the upper surface of the liquid.

Freeze cells in 50 mL cryovials without liquid nitrogen using this controlled-rate freezer to achieve high post-thaw recovery of biological samples.

Evaluating Post-Thaw Viability

To evaluate post-thaw viability with the refined cooling protocol, four vials were filled with 30 mL suspensions of Jurkat cells at a density of 107 cells/mL in RPMI-1640 (Thermo Fisher Scientific), containing 10% HyClone™ Calf Serum (Cytiva) and 5% DMSO, as well as 12 vials containing only medium and DMSO. These were placed and cooled in the VIA Freeze™ Quad freezer as per the protocol in Table 1 before being transferred to ultra-low temperature storage at -140°C.

After a minimum of 24 h storage, samples were thawed in a 37°C water bath for approximately 16 min and re-cultured.

Viable cell count through fluorescein diacetate (FDA) staining was assessed at 24, 48, and 72 h post-thaw using a Cytell Cell Imaging System (Cytiva). Cell functionality was also determined with a 4 h 30 min alamarBlue™ relative fluorescence assay using a GENios FL plate reader (Tecan), which provides a more in-depth analysis compared with viable cell number alone.

The samples were compared to an unfrozen control at all time points and experiments performed in triplicate.

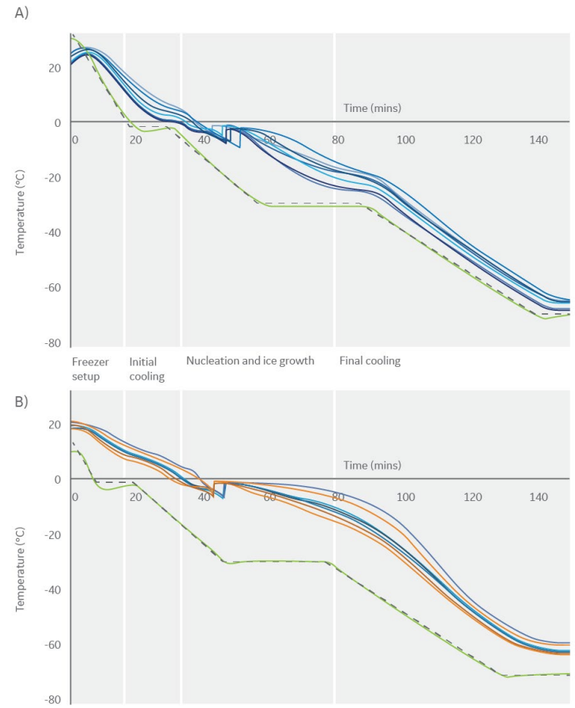


Fig 2. The thermal profiles observed during a 16-vial cryopreservation run, showing programmed cooling profiles (black) and freezer temperatures (green). (A) Thermal profiles observed for eight large-volume vials (blue) to evaluate inter-sample variability during cryopreservation. (B) Thermal profiles observed for two large-volume vials (vial one in blue, vial two in orange), each containing four thermocouples to evaluate intra-sample variability during cryopreservation.

Results For Cryogenic Processing

Thermal Variation During Cryopreservation

The optimized cooling protocol helped maintain consistency between samples, as demonstrated by minimal inter-sample differences during cooling events in the first thermal test (Fig 2A, on the following page). Some deviation did occur during nucleation; however, this is expected in all types of vial due to the stochastic nature of ice nucleation (2), and allowed for through the hold step at -30°C.

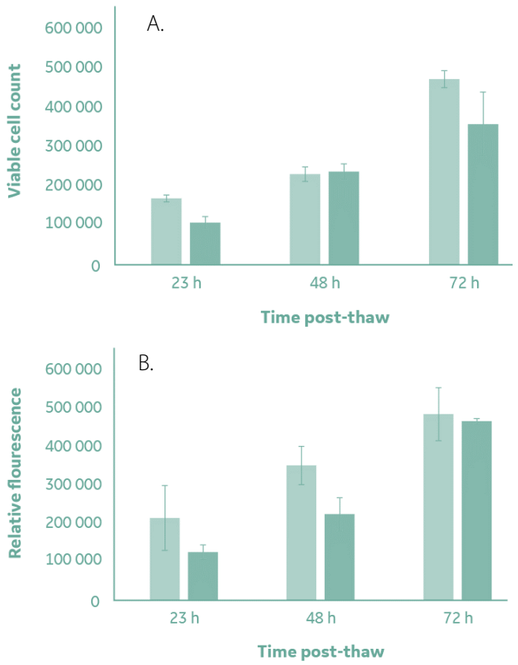


Fig 3. Analysis of cell viability and metabolism for samples frozen in large-volume cryovials (blue) compared to unfrozen control (gray). (A) viable cell count via FDA staining, and (B) metabolic activity read by alamarBlue™ relative fluorescence activity. Cells were thawed then cultured under normal conditions for 24, 48, and 72 h. Data is n=3 technical replicates ± one standard deviation of mean averages.

There was also minimal intra-sample thermal variation in the second thermal test (Fig 2B, on the following page). The variations seen within the two vials containing thermocouples is comparable to the intra-sample differences. These results suggest that the differences are a consequence of well-known intra-sample variation (1), as thermocouples are difficult to place in the exact same location in different samples.

The number of viable cells recovered after 24 h of culture post-thaw was 74% ± 16% compared to the unfrozen control (Fig 3A). This viable cell count is consistent with the expected impact of cryopreservation and culture interruption.

The subsequent strong growth in cell number is observed across all samples, with fold changes of 1.9 ± 0.3 and 3.2 ± 0.9 after 48 and 72 h of culture post-thaw respectively, compared to the 24 h time point. These results indicate that cryopreservation in vials using the optimized protocol does not inhibit proliferation and results in no noticeable delayed onset cell death.

Post-thaw, cryopreserved cells have ≤ 24 h delay on matching the metabolic activity of the unfrozen control, followed by a rapid recovery after ≥ 48 h (Fig 3B). This recovery period is also consistent with the expected impact of damage caused by cryopreservation and culture interruption. The alamarBlue™ data also supports the conclusion that, post-thaw, cryopreserved cells are healthy as they continue to metabolize and proliferate.

Conclusions On Cell Freezing In Large-Volume Cryovials

Despite the thickness of large-volume cryovials, the thermal tests demonstrate that effective cooling can be achieved with an LN_2-free VIA Freeze™ Quad controlled-rate freezer with a few simple adjustments to a standard cryopreservation protocol, such as lowering the start temperature and allowing for a thermal lag at key stages.

The optimized protocol outlined in Table 1 enables consistent cooling profiles and, while a thermal lag is apparent, tests indicate that there are no major inter- or intra-sample thermal variations. The samples can safely cool to below -60°C, a temperature suitable for biological cryopreservation, without incurring unexpected damage. Similar cooling profiles are known to be acceptable for T cell cryopreservation (3).

The biological test data demonstrates that interrupting the culture and cryopreserving samples using this protocol does not affect cell viability and metabolism. Cells recover from any delay in proliferation compared to unfrozen controls within 24 h post-thaw.

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Cryopreservation to Improve Cell Manufacturing and Biobanking

As Adoptive Cell Therapies and Stem Cell Therapies Come To Be Applied More Generally, Cryopreservation Technology Must Cope with Quality and Supply Challenges

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By Andy Tay, PhD

November 3, 2020 — Cell therapy is an emerging form of treatment that is poised to revolutionize medicine. Several chimeric antigen receptor (CAR) T-cell therapies have been approved by the U.S. Food and Drug Administration for treating leukemia, and at least 500 other studies using CAR T cells for cancer treatment are under clinical trials. A year ago, Japan's health ministry also approved the world's first use of corneal cells reprogrammed from induced pluripotent stem cells (iPSCs) for therapy,[1] paving the way for expanded use of iPSCs in clinics.

The process of cell manufacturing typically involves cell isolation and engineering before shipping cryopreserved cells from a centralized facility to clinics for infusion into patients. Technology for cryopreservation is hence critical to provide high-quality cells to achieve desirable clinical outcomes. Good materials and methods for cryostoring cells are equally pivotal to biobanking for maintaining human biospecimens such as tumor cells, umbilical cord blood, and tissue-derived stem cells, as well as excess engineered cells in cases when repeated doses are needed for efficacious therapies.

During the process of cryopreservation and thawing, cell viability can suffer due to reasons such as dehydration, toxic solute levels, formation of intracellular ice crystals, and osmotic stress.[2] The most common protocol for cryostoring uses dimethyl sulfoxide (DMSO),[3] a cell membrane–permeating cryoprotectant that reduces intracellular ice crystal formation. However, this comes with a trade-off as DMSO is known to induce oxidative stress and gene expression changes. This chemical can also damage organelles, cytoskeletons, and cell membranes, thus adversely affecting cell viability and functionality.[4] It has been reported that the cell viability of thawed CAR T cells can be as low as 50% after cryopreservation with DMSO.[5]

Recently, a team of scientists from the National Institutes of Health (NIH) led by David Stroncek, MD, found that DMSO-cryopreserved CAR T cells displayed similar expansion capacity, transduction efficiency, and clinical outcomes to their fresh counterparts—however, the cryopreserved cells exhibited greater mitochondrial dysfunction, apoptotic signaling, and cell damage. Stroncek and colleagues stressed the need to better assess the clinical impact of cryopreservation-related microdamage.[6] Previous reports had demonstrated that the in vivo therapeutic efficacy of cryopreserved natural killer (NK) cells is significantly lower than that of fresh NK cells.[7] A similar finding emerged when cryopreserved and fresh mesenchymal stem cells were compared for their ability to inhibit T-cell proliferation in vitro.[8]

"Cryopreservation is absolutely essential to allow all release testing to be performed and for shipping CAR T-cell products," argues Bruce L. Levine, PhD, a professor at the University of Pennsylvania. "There is a need for improved formulations with a lower percentage of DMSO that can enhance cell recovery and viability. DMSO-free formulations should also be considered for preserving CAR T cells."

Although the use of fresh cellular materials may be advantageous and help to achieve better clinical outcomes, it can make manufacturing and logistics much more complex and expensive. Currently, cell therapies such as CAR T-cell treatments and their associated healthcare costs can be as high as $500,000 per patient,[9] and medical literature has started referring to this problem as "financial toxicity." Therefore, there is a strong demand for improved materials and methods for cryopreservation, and better means to assess the biological and clinical impacts of cryostorage to make cell manufacturing and therapy more efficient.

Emerging Materials for Cryopreservation and Thawing

"With widespread application of cell therapy, cells have to be maintained at high viability and function at ambient, hypothermic, or cryogenic temperature for convenient and wide

distribution to end users," notes Xiaoming He, PhD, a professor at the University of Maryland. "Some ways to tackle this challenge include replacing toxic chemicals like DMSO and glycols with biocompatible materials like sugars as cryoprotective agents, encapsulating cells in hydrogels, and using nanomaterials."

Inspired by the ability of tardigrades, or water bears, to survive freezing, the team led by

"It is also crucial for biological materials to retain high viability, structural integrity, and functionality after cryopreservation and thawing."

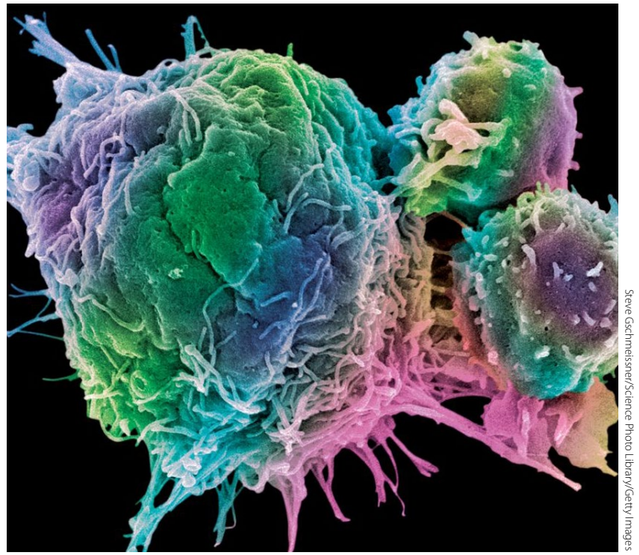
Xiaoming He conjugated trehalose, a natural sugar that disrupts intracellular ice crystal formation, to nanoparticles that can be readily endocytosed by human stem cells. This technology overcomes the inability of trehalose to permeate the cell membrane and enables intracellularly available trehalose to confer cryoprotection.[10] The same team also designed cold-responsive nanoparticles that release trehalose intracellularly upon cooling, and offers the additional benefit of skipping a cell washing step that can lead to cell loss.[11]

A group of researchers at the University of Warwick led by Matthew I. Gibson, PhD, also demonstrated that extracellular deposition of naturally occurring type III antifreeze proteins significantly improves the post-thaw recovery of cells seeded in a monolayer.[12] Recently, Anne Meddahi-Pellé, MD, PhD, and her team from the University of Paris synthesized dextran-based hydrogels that demonstrated comparable cryoprotective ability as DMSO, without the side effects, albeit in human cell lines.[13]

According to David Stroncek from NIH, there is also a strong need for better thawing technology. "Thawing technology is important because thawing cells too slowly can damage cells, and once cells are thawed, DMSO is cytotoxic," he points out. "The thawing process varies from center to center, but most protocols involve rapid thawing of the cells in a water bath with manual manipulation of the cells."

Together with colleagues from China, Xiaoming He synthesized magnetic nanoparticles[14] and made use of magnetic induction heating to more uniformly thaw stem cells. The nanomaterials provided a spatially homogenous warming rate during thawing, enabling faster and more uniform melting of ice crystals.

Remarkably, stem cells that were thawed using the nanoparticle technique exhibited higher viability and proliferative capacity while suffering minimal damage to their organelles, compared to their counterparts thawed using water baths. Crucially, the stem cells also better retained their ability to differentiate to different lineages, an important biological criterion for the use of stem cells in regenerative medicine. A similar result was reported by John Bischof, PhD, and his colleagues at the University of Minnesota.[15] They made use of inductive heating of magnetic nanoparticles to minimize thermal mechanical stresses and improve cryopreservation of cells and tissues.



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When chimeric antigen receptor (CAR) T cells are thawed after cryopreservation, quality assessments go well beyond questions of viability. A comprehensive panel to investigate the impact of cryostoring cells on CAR T cells should include general tests (such as proliferation, metabolism, and gene expression changes) and application-specific tests (such as differentiation potential and cytotoxicity).

Assessing the Impact of Cryopreservation

"It is also crucial for biological materials to retain high viability, structural integrity, and functionality after cryopreservation and thawing," maintains Xiaoming He.

Typically, assays measuring cell viability and metabolism are used to evaluate the impact of cryopreservation. However, these metrics provide little information about whether the critical polyfunctionalities of cells are affected.

For instance, while cell viability after cryopreservation may be high, the cytotoxic functions of CAR T cells or the regenerative capacity of stem cells may be altered. Hence, researchers are starting to assess the biological and clinical impacts of cryopreservation more comprehensively.

Gene expression change is an important metric because it provides a global view on the different gene pathways that are affected by cryopreservation and the extent to which they are being affected. A team led by Qing Ye, MD, PhD, from the Affiliated Hospital of Nanjing University Medical School found that different tissues and cells are affected differently by cold ischemia,[16] which occurs to biological materials

when they are denied their blood supply and chilled, until they are warmed by having their blood supply restored. The integrity of medium and long genomic DNA and of RNA was impacted by the length of cold ischemia time, with nucleic acids found in skin tissues being most resistant to degradation after isolation.

Aifen Lin, PhD, and her colleagues at the Wenzhou Medical University, examined the effects of –80°C storage on the quality of gastric tumor tissues.[17] They found that the impact of storage duration on RNA integrity was minimal, but the composition of the tissue specimens, that is, the ratio of tumor to stromal cells (in particular, those with greater percentage of tumor cells), had better RNA quality after extraction. These results demonstrate the importance of performing quality control to accurately assess the impact of cryopreservation on gene expressions of different cell types used for therapy.

Application-specific assays are also crucial to determine whether cryopreserved cells are going to function therapeutically. For instance, Xiaoming He and his team administered several tests, including assessments of stem cell differentiation potential, to evaluate the impact of their magnetic nanoparticle heating technology.

For CAR T cells, a combination of in vitro and in vivo assays is necessary to examine the effects of cryopreservation.[18] Some of the in vitro assays include CAR T-cell transwell migration, cytokine production, and cytotoxicity measurements. Common in vivo tests include CAR T-cell infiltration into tumor tissues (number and spatial distribution) and survival profiles of animal models and humans. A comprehensive panel to investigate the impact of cryostoring cells should include general tests (such as proliferation, metabolism, and gene expression changes) and application-specific tests (such as differentiation potential and cytotoxicity).

Conclusions

The field of cell therapy is evolving rapidly with new therapeutic targets and with better techniques to transfect cells[19] and enhance safety.[20] As the population ages, the demand for cell-based regenerative medicine, biobanking, and tissue transplants is also likely to increase. These trends will intensify the need for better ways to characterize the impact of cryopreservation technology to improve cryostoring and thawing. Innovations in cryopreservation will also benefit biomedical research by enhancing current protocols and tools to preserve organoids[21] and xenografts, which are becoming more popular in drug screening and basic science research.

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