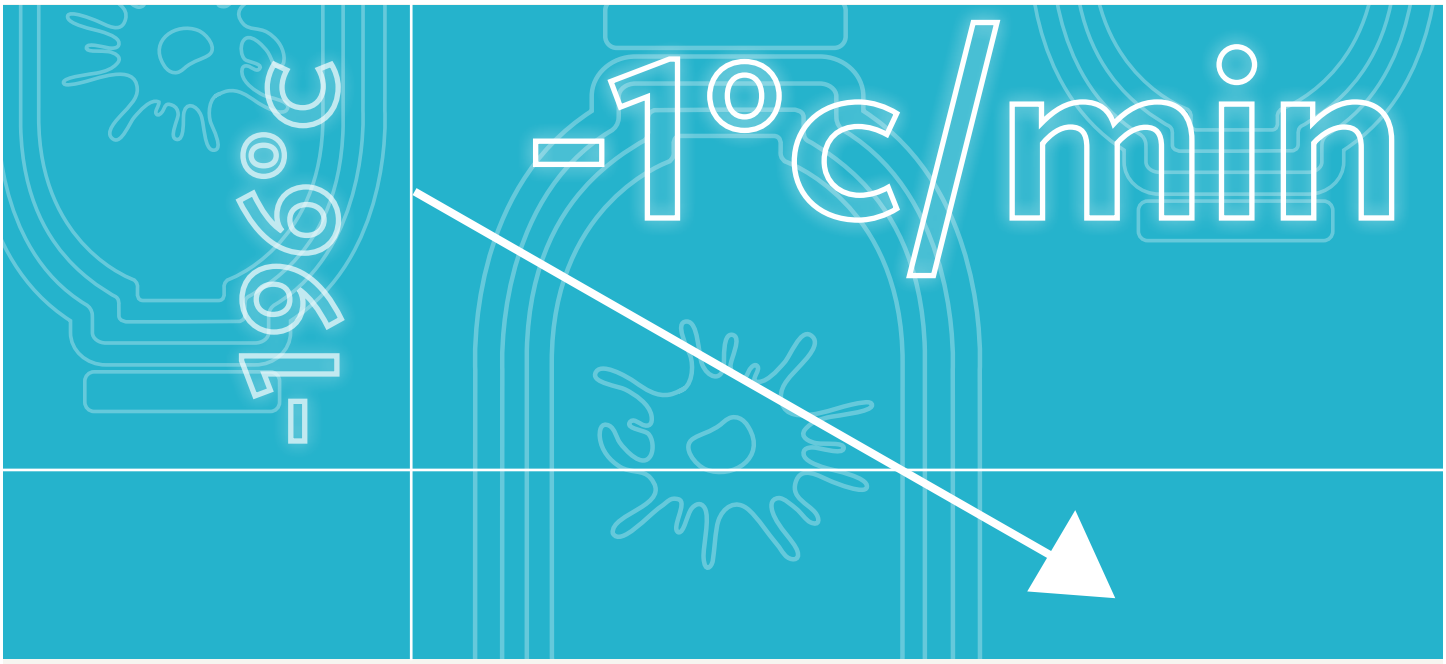

Controlled-Rate Freezing of Cells During Ultra Cold Transit

By François Hamy, Ph.D, Fisher BioServices

Fisher BioServices

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Content

About the Author

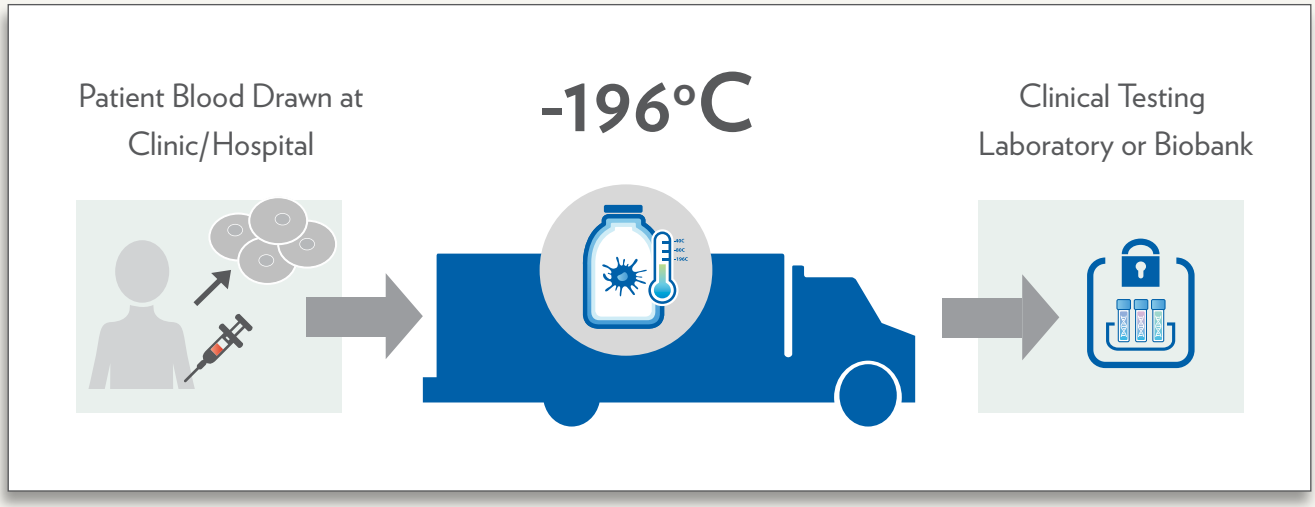
Dr. François Hamy manages FisherBioServices' laboratory operations in Basel, Switzerland. In this role, he is responsible for processing and analyzing samples as well as managing the Biomarker Laboratory, which uses extensive genomics, proteomics, phenomics, and metabolomics studies to support clients' needs in biomarker development. In addition, François is one of the major contributors to the technical innovation team at Fisher BioServices that focuses on supporting customers' project needs and challenges. This often includes designing and developing custom solutions for biobanking, clinical trial materials management, as well as ultra cold chain logistics.

Dr. Hamy has served 11 years as a research group leader in virology/oncology in the pharmaceutical industry, including Novartis and Sanofi Aventis. He also has six years of experience in leading a virology/oncology diagnostic company. He received his PhD training in molecular biology at the University of Lille, France, and completed his Masters Degree in Pharmacology & Drug Design from the University of Nottingham, U.K.

Abstract

Patient cells that are isolated for development into a therapeutic product must be processed quickly or they lose their integrity and therapeutic value. Cells that cannot be processed immediately must be cryopreserved at -195°C in liquid nitrogen.

However, to ensure viability and integrity upon thawing, freezing down to -195°C must be performed at a specific cooling rate, which requires equipment that exceeds the financial resources of most clinical sites. Fisher BioServices presents an inexpensive and innovative solution that allows staff at any clinical site to draw and process patient blood and place the resulting peripheral blood mononuclear cells (PBMCs) in a specialized shipper that will freeze the cells to -195°C. This solution utilizes a novel device and configuration that is capable of cooling at a rate between 0.5°C and 2.0°C per minute, reaching –195°C over a span of three hours as the cells are in transit to a laboratory or biorepository storage facility.



Introduction: The Problem

Cell-based therapies, regenerative medicine, vaccines, and other biological materials frequently require processing and administration to the patient within an extremely short timeline (24 hours).

The alternative is to preserve the cells at cryogenic temperatures (below -150°C) to maintain their live properties until the appropriate time. Ideally, cells and living organisms are preserved in liquid nitrogen (LN_2), at -196°C, at which temperature all biochemical activity ceases.

The cryopreservation of cells must be performed in a specific manner in order to recover the critical properties of those cells upon thawing. This involves use of a well-defined cryoprotective medium to prevent crystallization and destruction of cells during the freezing process, and a cooling rate of between -0.5°C and -2.0°C per minute (with a target cooling rate of -1.0°C per minute) to prevent temperature shock. This controlled rate, combined with the protective medium, leads to optimal viability of the cells upon thawing.

How Much does it Cost?

Cryopreservation is a costly process. Controlled-rate freezing equipment is expensive and requires a source of LN_2 as coolant. An LN_2 tank is also needed for storage of the materials once they are frozen. Few clinical sites, where patient samples are drawn, have the equipment needed to freeze patient cells directly to cryogenic temperatures.

The current, less costly alternative to controlled-rate freezing units are the passive containers that provide insulation and controlled cooling within a -80°C ultra-low mechanical freezer. The vials are placed in the container, which is then placed in the ultra-low freezer for about 16 hours, until the cells reach -80°C. These devices (such as the Mr. Frosty® container, manufactured by Thermo Scientific Nalgene™ and the CoolCell® device manufactured by BioCision) are widely used, accepted in the research community, and are economical. However, these devices require access to an expensive mechanical -80°C freezer.

Introduction: The Problem

These containers also subject the vials to an extra step in handling during transfer from the -80°C container directly into a LN_2 environment. This transfer results in an abrupt drop of more than 100 degrees in temperature. In addition, the time required to cool material to -80°C in an ultra-low freezer plus transfer to the LN_2 tank is greater than 16 hours, which does not allow for a delay in processing. Further, if the LN_2 tank to be used for long-term storage is not onsite, then the vials must also be shipped to the storage site.

What About Shipping?

The pharmaceutical industry primarily uses dry ice to ship frozen materials; dry ice sublimes from solid to CO_2 gas phase while maintaining a local temperature of approximately -80°C. As a result, the International Air Transportation Association (IATA) classifies dry ice as dangerous goods. In addition, the amount of dry ice needed to maintain temperatures results in bulky and heavy packaging, contributing to higher transportation costs. Further, the rate at which dry ice sublimes allows maintenance of -80°C for only a few days.

In contrast, liquid nitrogen shipping containers will preserve materials at the optimal temperature of -196°C during transit, and can maintain that temperature for more than two weeks. Another advantage is that LN_2 is not considered a dangerous material by the IATA.

Objectives

We proposed a freeze-and-ship technology that would not require, costly investments at the investigating site and would provide immediate cryogenic freezing of the cellular material during transfer to the destination laboratory or biobank.

To develop an acceptable solution, step-down freezing and concurrent shipping must provide optimal preservation of patient cell integrity and post-thaw viability for downstream processing and administration. This technology had to be affordable, verified to produce a cooling rate of -1°C per minute, and be compatible with ultra cold chain logistics.

Methods

We developed numerous prototypes to determine the thermal resistance/conductivity criteria required for the insulation material.

The insulating material had to show specific resistance properties to very low temperatures in LN_2 (-196°C) when installed in the selected shipper. In addition, this material had to provide a highly reproducible and uniform -1.0°C per minute cooling rate, and be suitable for vial contact. A high-density foam that met the criteria was eventually selected.

Once a suitable material was located, the position of the vials within the insulating material that would allow a -1°C per minute cooling rate had to be determined. The cooling rate is a function of the distance between the vial and the LN_2, and would also ultimately determine the number of vials that could be placed in the freeze-and-ship container.

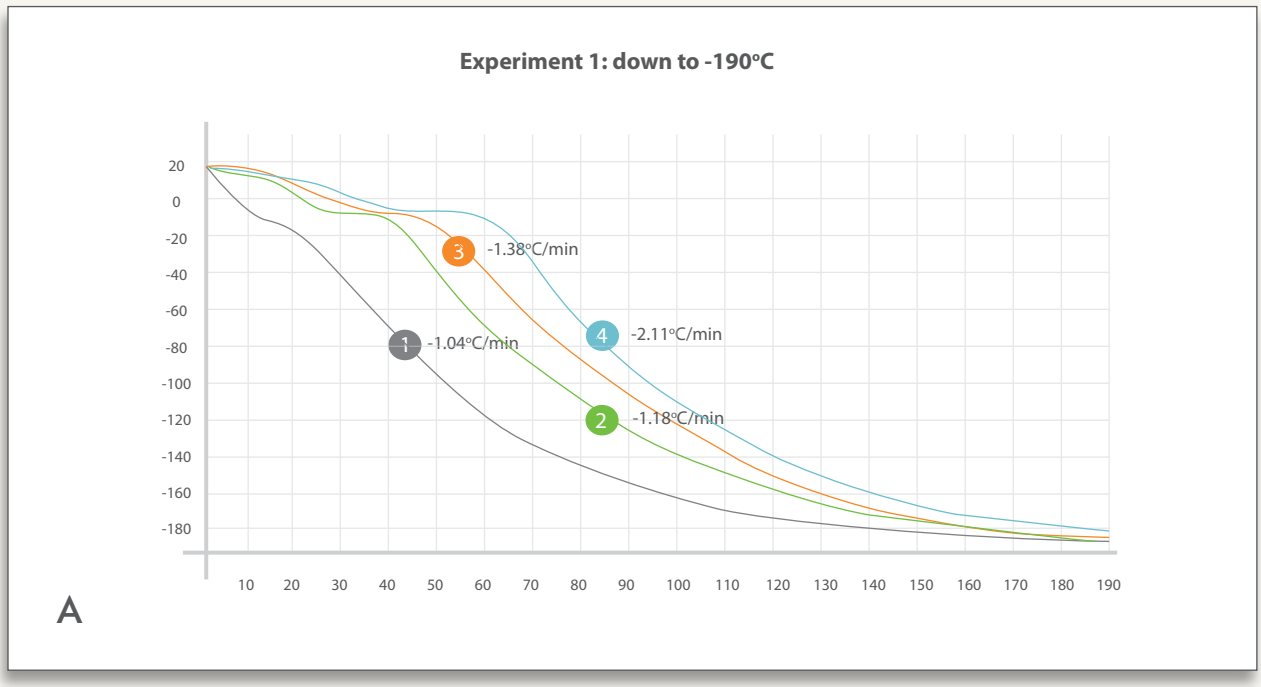
A temperature recording device (certified ELPRO system) with four temperature probes was used to measure the cooling rate at different locations.

The probes were first placed in cryotubes containing a cryoprotective solution, to mimic the conditions of freezing cells in suspension. The cryotubes were then inserted at four different locations in the foam (Figure 1).

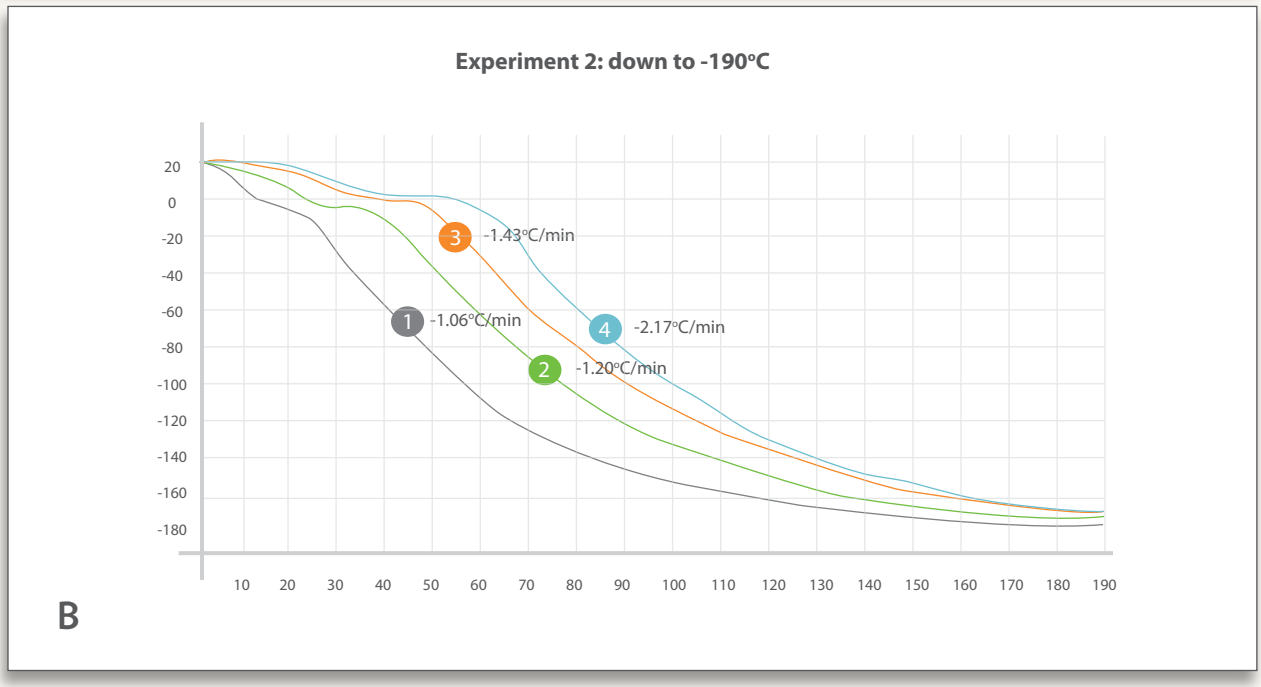
Figure 1: Placement of the temperature probes in the foam

Methods

Multiple experiments using different probe locations were run, to determine the cooling rate relative to their location in the foam. The results appear in the charts in Figure 2A, 2B, 2C, below.



A



B

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Methods

The data shows that the temperature of all vials in the container reached -190°C in approximately three hours (180 minutes), compared to 16 hours to reach -80°C using the Mr. Frosty®. The cooling speed for the four different locations was similar for the three experiments, demonstrating that the controlled cooling rate was highly reproducible.

Also, as expected, the cooling rate between the four locations varied with the distance from the LN_2; the closer to the LN_2, the faster the cooling rate. Locations 1, 2, and 3, which were more centrally located in the three different configurations, all delivered a cooling rate between -0.5°C and -2.0°C per minute, while location 4, which was peripherally located in the three different configurations, resulted in a temperature drop of -2.13°C per minute and exceeded the acceptable cooling rate.

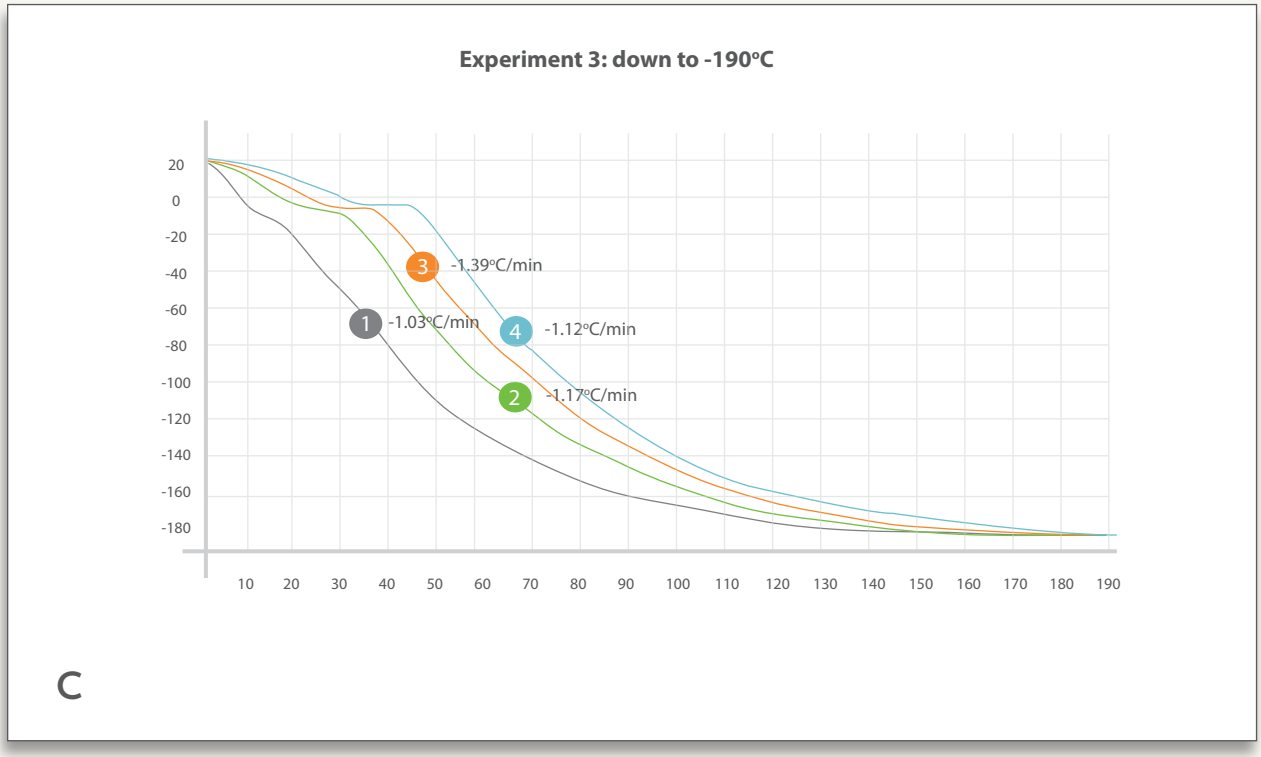

Figure 2: Cooling rates recorded for the four probe locations under three different configurations within the foam.

(A) Cooling rates recorded in configuration 1

(B) Cooling rates recorded in configuration 2

(C) Cooling rates recorded in configuration 3

Methods

To test the solution, we compared the viability of cells that were frozen and then thawed using the Mr. Frosty® system with the viability of cells frozen in our freeze-and-ship container. The experimental protocol used to test cell viability is illustrated in Figure 3, below.

Blood was collected from healthy donors and PBMCs were prepared using a cell isolation tube containing ficoll gel. After PBMC separation and washes, following a standardized protocol, cells were suspended in a standardized cryoprotective medium (Cryostor-5) or in phosphate-saline buffer (PBS). PBMCs were then aliquoted into cryotubes and placed in either the Mr. Frosty® or in the freeze-and-ship device. The Mr. Frosty® was then placed in a -80°C freezer and the vials later transferred to a LN_2 storage tank, according to manufacturer's recommendations. For our freezing/shipping container, the cryotubes were inserted into the foam insulation and the foam was placed directly into the -196°C LN_2 dry shipper.

After one week of storage at -196°C in a vapor-phase LN_2 tank, cells from both freezing conditions were thawed and cultured according to standard protocols (24 hours, 37°C, 5% CO_2). Cells were subsequently activated with phytohemagglutinin (PHA) or Interleukin-2 (IL-2) as illustrated in Figure 3 below, incubated for an additional 16 hours and directly assayed for cell viability (MTT colorimetric test, Promega) and for cell proliferation (Click-it EdU test, Invitrogen).

Cell viability is the primary criteria for determining cryopreservation efficiency. Since reduced cell mortality can lead to diagnostic errors or sample loss, it was essential for us to establish that our solution preserved cell viability and integrity at the highest possible levels.

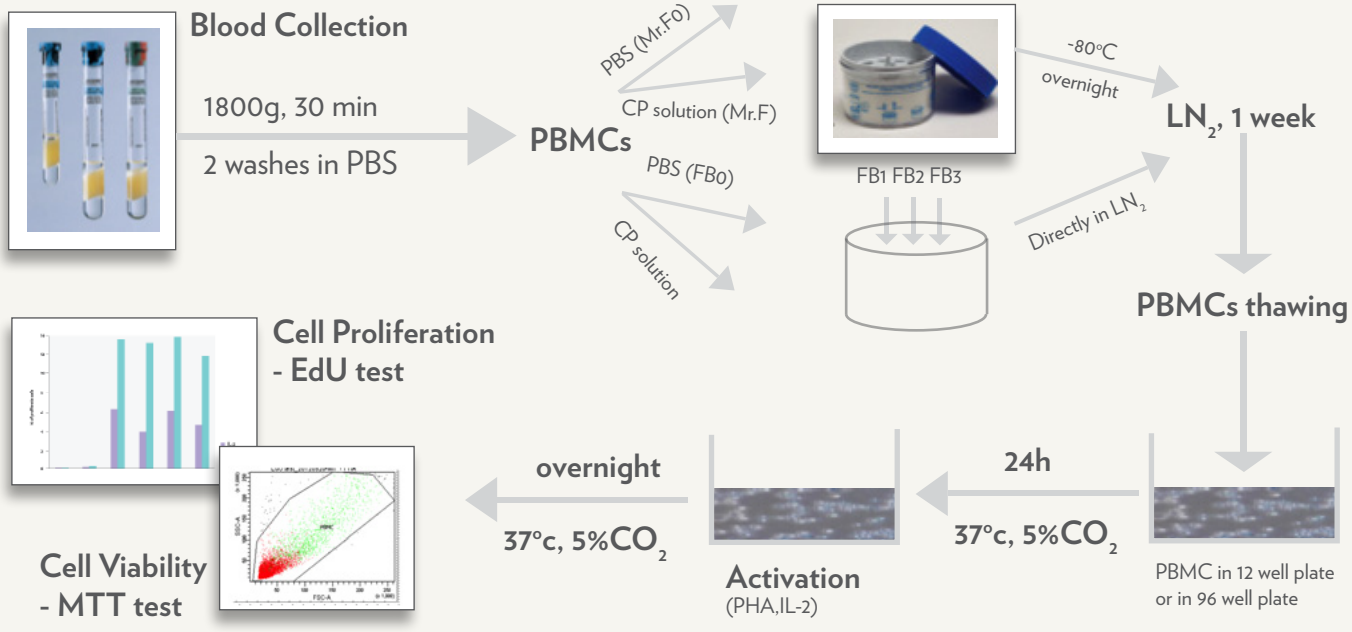

Figure 3: Protocol for testing cell viability after freezing by Mr. Frosty® or by the freeze-and-ship device.

Results

Results from the viability test are depicted in Figure 4, below. As expected, cells frozen under both conditions without cryoprotectant failed to recover. However, the viability of the cells that were cryogenically frozen at the three different locations of our device (FB1, FB2 and FB3, Figure 3) was highly comparable to the viability of the cells frozen with the Mr. Frosty® freezing protocol both in terms of cell viability (IL-2) and also as activated by a mitogenic agent (PHA). These results demonstrate that, despite a slight variation in cooling rate at the different vial locations in our device, neither cryopreservation efficiency nor cellular recovery was affected.

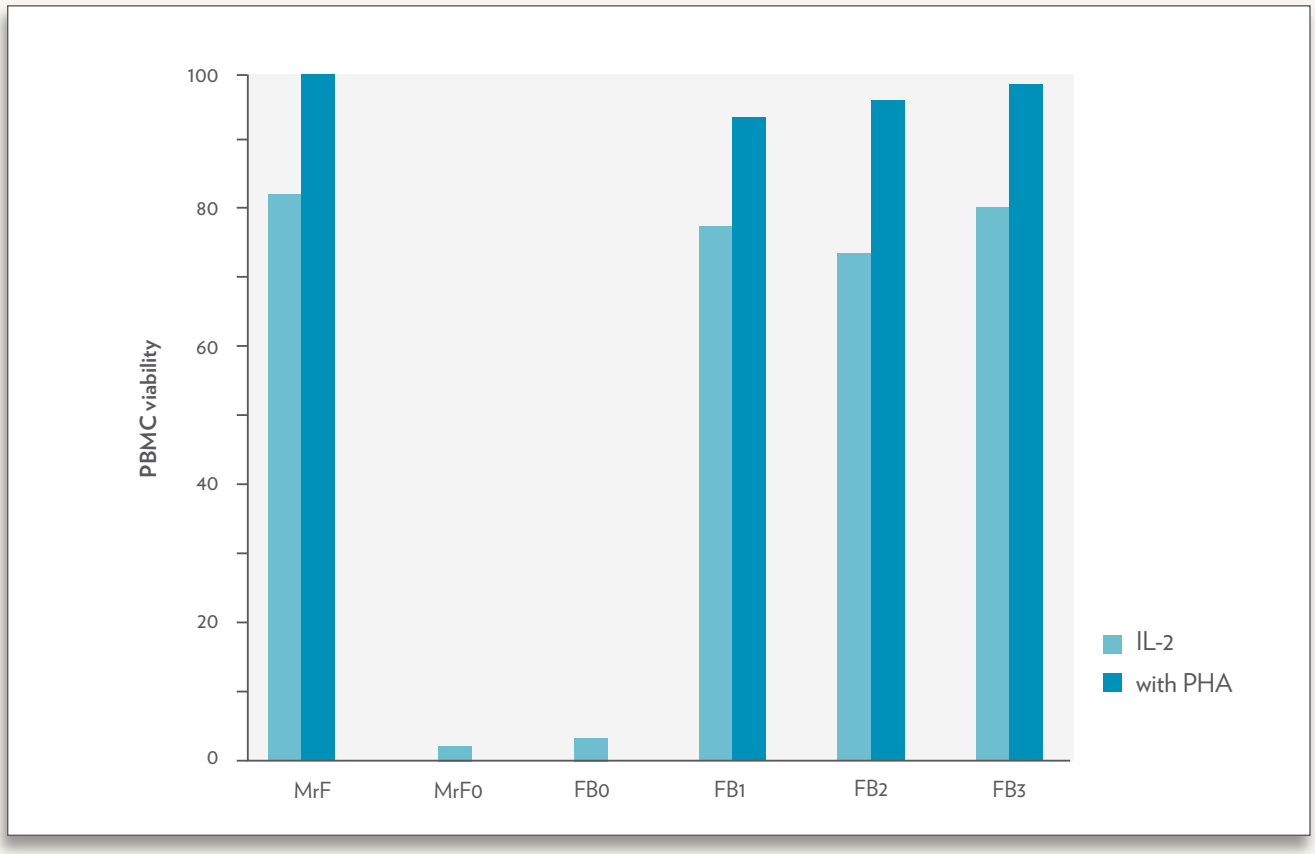

Figure 4: Results of cellular viability experiments.

Results

To illustrate a more precise recovery of the immune-phenotypic properties of PBMCs frozen under the two conditions, we next performed a flow cytometry experiment, using the incorporation of EdU into newly synthesized DNA, thereby indicating a proliferation rate of PBMCs. The results of this experiment are depicted in Figure 5, below.

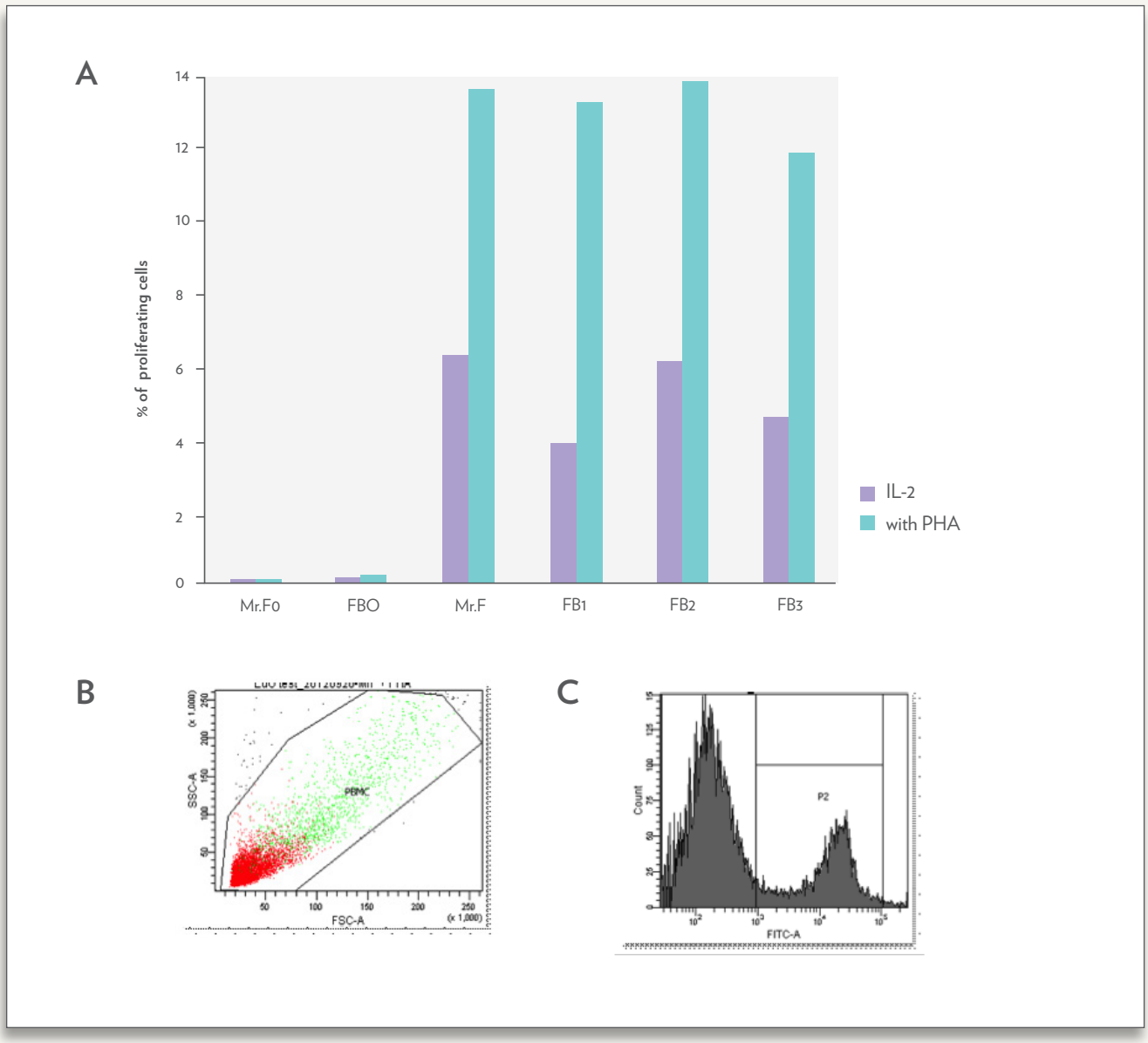

Figure 5: Results of cellular proliferation experiments

(A) % Cell Proliferation of all tested samples.
(B) Dot plot depicting the cell population from FB2 by size and complexity.
(C) Histogram showing the proliferation of cells in FB2, which are characterized by the incorporation of EdU.

Conclusions and Applications

We investigated a means of using an inexpensive, passive controlled cooling devices to cryopreserve cells in LN_2, all the way to -196°C, rather than in two stages (freezing to -80°C in a mechanical freezer followed by transfer to a LN_2 tank, which results in an abrupt temperature drop). We sought a solution that would result in the optimal combination of reducing cost and protecting cells from the sudden >100°C drop in temperature.

Our innovative freezing solution provides a controlled rate of temperature drop to a cryogenic temperature range (-196°C) in a single step, without the expense of a programmable step-down freezing unit, a mechanical ultra-low temperature freezer, or a full size LN_2 storage tank.

We determined that our freeze-and-ship device was as easy to use as the Mr. Frosty®, while also performing as well as the current devices on the market as determined by post-thaw cell viability and recovery of cell proliferation and immunophenotypic characteristics. Our freeze-and-ship solution offered other advantages, as summarized in Figure 6, on the next page.

Conclusions and Applications

Freeze-and-Ship Device	Mr. Frosty®	CoolCell®
No additional chemical entities. No maintenance.	Uses 100% Isopropanol On-going purchase, maintenance, hazardous waste.	No additional chemical entities No maintenance. No fluids.
No fluids. No cooling required by site staff. No hazardous waste.	Due to isopropanol degradation; Pre-cooling of hazardous alcohol in refrigerator.	No additional chemical entities. No maintenance.
Ready to use.	Takes >1 hour for device to return to room temp.	Ready to use.
No -80°C freezer or LN_2 required: Fisher BioServices provides the containers pre-charged with LN_2.	Require isopropanol, a -80°C freezer, and a -196°C LN_2 storage tank.	Require a -80°C freezer and a -196°C LN_2 storage tank.
Minimal handling Insert the vials into the foam container and place in the liquid nitrogen vessel.	**Several handlings** Replace isopropanol every five uses in the container. Place the container in -80°C freezer, remove vials and transfer into dry ice shipper and/or LN2 tank.	**Some handling** Place the container in -80°C freezer, remove and transfer into dry ice shipper and/or LN2 tank.
Allows delayed processing or administration.	Does not allow delayed processing or administration.	Does not allow delayed processing or administration.
3 hours to freeze down to -196°C.	16 hours to freeze down to -196°C.	3 hours to freeze down to -196°C.
Freeze-and-ship container.	Freezing container.	Freezing container.
No hidden cost; no investment, maintenance, or energy costs for -80°C or LN2 freezers.	~$350/year maintenance per unit Change isopropanol every five uses + hazardous waste disposal.	No hidden cost.

Figure 6. Comparison of the three controlled-rate cooling devices.

Conclusions and Applications

We set out to find a cost-effective means of allowing the simultaneous transfer and progressive cooling of patient PBMCs and other cell types at clinical sites that do not have ultra-low temperature freezers or LN_2 handling capabilities.

The freeze-and-ship device requires no investment by clinical sites or doctor's offices in ultra-low temperature (-80°C) freezers (or the passive cooling devices like the Mr. Frosty® used to provide step-down cooling within the freezer), infrastructure for handling and storage of LN_2, or other controlled-rate cryogenic freezing equipment.

With only one or two working days notice, Fisher BioServices can deliver a freeze-and-ship device, precharged with LN_2 and at the needed temperature, to a clinical site in time for a patient appointment. The patient sample is processed as usual at the site laboratory, and the vials placed in the freeze-and-ship container according to the instructions provided. The container is then shipped to the destination laboratory or biobank as applicable.

Fisher BioServices can provide shipping arrangements to third parties, provide training to clinical site staff if needed to ensure that best practices are followed in the use of the freeze-and-ship device, and arrange for the return of the device to Fisher BioServices for reuse. If you'd like to learn more about this study and discuss how our cold chain logistics solution can support your project in transporting critical biological materials in ultra cold temperatures, please contact us directly using the information on the next page.

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