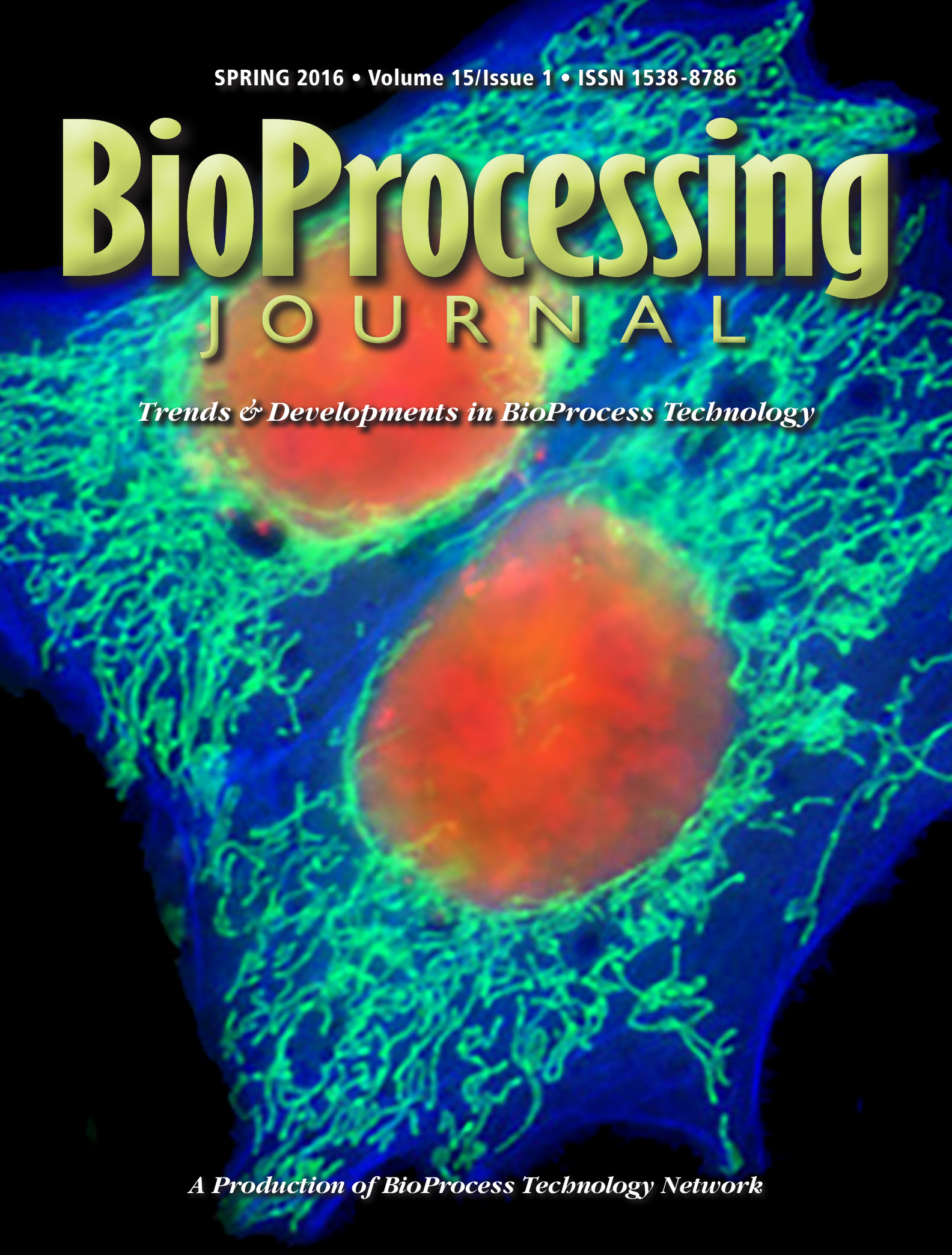
SPRING 2016 • Volume 15/Issue 1 • ISSN 1538-8786

BioProcessing JOURNAL

Trends & Developments in BioProcess Technology



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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Development and Assessment of a Novel Device for the Controlled, Dry Thawing of Cryopreserved Cell Products

By John M. Baust, William L. Corwin, Kristi K. Snyder, John G. Baust, and Robert G. Van Buskirk

Abstract

While playing an integral role in biotechnology and medicine, cryopreservation (CP) is often viewed as a "simple tool" and is overlooked as a critical and evolving component of cell and tissue bioprocessing. Despite this, cryopreservation serves as an enabling technology in numerous areas including the latest cell therapies. For example, over one third of the cells used in clinical trials are cryopreserved using the traditional methods, which in many cases yield suboptimal outcomes. Further, researchers still rely on the assessment of cell survival immediately post-thaw (within a few hours), and fail to account for the impact of cryopreservation-induced delayed-onset cell death (CIDOCD) which continues to impact survival from hours to days post-thaw. Interestingly, despite the fact that CP research remains in a growth phase that focuses on the role of the cellular molecular response to CP stress, these discoveries, and the resultant paradigm shift, have yet to filter into mainstream utilization.

Given the crucial role of CP, coupled with other challenges needed to keep pace with modern biomedicine, we have embarked on a goal of developing a novel device and protocol designed to enable rapid, controlled, multi-sample, and high-throughput thawing in an effort to improve overall sample viability and function post-thaw. We have achieved this with the development of the SmartThaw™ device. Studies were conducted using human prostate cancer cell (PC-3) and human mesenchymal stem cell (hMSC) samples cryopreserved using standard, controlled-rate freezing protocols and then thawed with SmartThaw. Post-thaw survival results were equivalent or improved, as compared to traditional water bath approaches, depending on the freezing media utilized. All SmartThaw experiments were achieved in a clean, dry, sterile field with real-time monitoring of the sample thaw thermal profile.

Study results suggest that SmartThaw outperforms traditional methodologies. Importantly, these investigations are providing new technologies and direction that are built on a cell/molecular foundation that helps accelerate research, technology, and procedure development initiatives in which CP serves as an enabling component. Further, improvements in standardized sample thawing devices and protocols may pave a path for increased use of cryopreserved cells in clinical applications that enable improved post-thaw viability, homing, biodistribution, and engraftment.

Introduction

The cultured cell has emerged as a critical link in translating research in the various fields of biotechnology and biomedicine. An unprecedented increase in the demand for cultured cell products is now driven by the growth in biopharmaceuticals, cancer research, cell and gene therapy, and stem cell research. In the discovery sciences, high-throughput screening (HTS) using cultured cells provides an important tool for the drug discovery and drug toxicity testing fields as cell-based assays can be used for the identification of both desired and unintended drug effects. Improvements in cell-based platforms have become increasingly essential with a continued push to reduce the reliance on animal models. Properly selected cell models can provide more relevant *in vivo* biologic data than other assay options, and thus can reduce wasted time, money, and effort during discovery while limiting late stage attrition.

To see the impact of cell-based products on the clinical sciences, one needs to look no further than the cell therapy arena. Progress in the field of stem cell biology has opened the potential of new therapeutic options for a number of diseases.[1–5] With the advent of new techniques such as nuclear transfer-derived embryonic stem cells (NT-eSC) and induced pluripotent stem cells (iPSC) entering clinical trials, stem cell technologies are now translating into promising clinical therapies.[6,7] As a result, cell therapy is now being used worldwide to treat diseases ranging from cancer to diseases of the eye. In the United States, there are over 400 stem cell trials underway using mesenchymal stem cells (MSCs) alone.[2–4,8,9] We recently entered a new chapter in stem cell therapy transplant

technology where iPSCs are being used in the first clinical trial for treating age-related macular degeneration.[10–12] While iPSCs have proven valuable for disease modeling, drug testing, and *in vitro* toxicology, this first clinical trial hints at the real potential of iPSCs. While these newer stem cell technologies require further study and validation for practical clinical application, procedures and biological products utilizing adult stem cells, such as human hematopoietic stem cells (hHSC), are currently in clinical use.[13] Yet as reviewed recently, issues remain such as formulating regulatory guidelines as well as safe and efficient manufacturing procedures.[4, 9, 13]

Cryopreservation

Implicit in the mainstream utilization of cell-based products, including the cell therapy arena, is cryopreservation (CP). Currently, about one third of the MSCs used in clinical settings experience at least one round of cryopreservation.[3, 4, 14, 15] Cell CP is necessary to retain viability and functionality for extended periods of time and for the subsequent delivery of cellular products for "on-demand" utilization.[16] Numerous cell therapies and clinical trials rely on the use of biologics that have been cryopreserved.[5, 7, 8, 17–19] While the use of CP has become ubiquitous in many research and therapeutic areas, it remains a suboptimal process associated with distinct limitations and the potential for deleterious downstream effects on cell samples.[20–22] For many cell types, current CP and subsequent cryothawing protocols rely on the cell appearance immediately post-thaw as an indicator of viability. But when the cells are examined 1–3 days post-thaw, samples often experience significant loss and/or compromised function.[23–26] Sensitive cell types like stem cells may have cell loss rates >50% along with reduced metabolic response.[15, 21, 23]

Standard CP is distilled down to the suspension of a sample in a solution that contains a cryoprotective agent, most commonly dimethyl sulfoxide (DMSO), along with the application of a slow, controlled cooling rate to achieve an acceptable level of post-thaw viability and functionality. However, it is important to recognize that the cryopreservation process consists of five stages, all critical elements that can impact outcomes including: (1) isolation; (2) cell configuration; (3) freezing rate paradigm; (4) cryopreservation solution; and (5) thawing paradigm. While current CP processes provide a level of effective sample freezing, there are a number of issues associated with the process that make it less than optimal.[27, 28] Issues include: (1) extended processing times of 3–6 hours; (2) limits on sample container volume and configuration (*e.g.*, bags, vials, plates); (3) limits on available sample volume quantities; and (4) compromise of both sample quality and downstream function. Further, post-CP sample processing often creates a lengthy delay between thawing and when the material is actually used for the intended investigations.[22, 24–26] The delay further adds to the molecular-based stress response of cells to the CP process that results in reduced sample quality and quantity post-thaw.[25, 26]

The ultimate goal of CP should be to develop a program that integrates all five elements and results in optimal post-thaw viability, functionality, homing ability, biodistribution, and long-lasting engraftment potential. Interestingly, while a tremendous amount of effort and financial resources are invested in sample freezing every year, little attention is paid to the *thawing process* that is needed for the end-use. As with freezing, the thawing process has a tremendous impact on product quality and downstream utility.[29–31] Today, the gold standard for thawing samples used within the cell therapy and research community (whether in vials, bags, straws, or other formats) is a warm (37 °C) water bath. It works well, but there are a number of issues associated with the process that make it suboptimal. Sterility, which is paramount in clinical settings, consistency, controllability, documentation, and cleanliness are all issues that plague current thawing practices. It is imperative that CP technology catch up and develop new and cost-effective devices that support controlled, consistent, multi-sample, rapid, "hands-off" thawing of frozen samples (*e.g.*, cells, tissues, gametes [for IVF applications], *etc.*) compatible with both research and clinical settings.

We have developed a unique device, named the SmartThaw™ (CPSI Biotech, Owego, NY), for the programmable and documentable thawing of cryopreserved cells and tissues.[29] SmartThaw can thaw samples frozen in most standard formats (container types, brands, and volumes) including cryovials, freezing bags, and ampoules. One principle guiding the development of SmartThaw is to achieve the best preservation outcome by integrating each stage of CP into an optimized program.

In this study, we evaluated SmartThaw for its ability to thaw cryopreserved samples in standard cryovials and freezing bags. Assessment of thermal profiles during thawing, plus cell survival and repopulation post-thaw were conducted and compared to the results achieved with the time-proven 37 °C water bath protocol. The data presented herein demonstrate the ability of SmartThaw to consistently thaw cryopreserved samples in a user-friendly, hands-free, clean, and dry environment while attaining equivalent or enhanced cell survival and recovery.

Methods

Cell Culture

Human mesenchymal stem cells (hMSC) and prostate cancer cells (PC-3) from ATCC were maintained under standard culture conditions (37 °C, 5% CO_2/95% air). The hMSCs were kept in mesenchymal basal medium supplemented with a low serum growth kit, gentamicin, amphotericin B, penicillin, and streptomycin; while the PC-3 cells were maintained in RPMI media with 10% serum and 1% pen/strep (ATCC). The cells were propagated in Falcon T-75 flasks (Corning) and the media was replenished every two days.

Cryopreservation

Cell culture medium was replaced with fresh growth medium 24 hours prior to freezing. The cell monolayer was detached by the addition of TrypLE™ (Thermo Fisher Scientific) for 5 min at 37°C. After the 5 min incubation period, 3 mL of growth medium was added to the cell suspension to inhibit enzymatic dissociation. The cell suspension was divided equally and pelleted at 100×*g* for 5 min. The supernatant was decanted, and the cell pellet was resuspended at 1×10^6 cells/mL in various CP solutions at 10 °C. The CP solutions used were either Viaspan® (Teva Pharmaceuticals) or Unisol™ (Tissue Testing Technologies LLC) culture media supplemented with 5%, 10%, or 15% DMSO (Sigma-Aldrich). Prior to use, the Unisol solution was supplemented with 3 mM glutathione as recommended by the manufacturer. The cell suspensions were then placed into 2 mL cryovials (1 mL/vial) and CS 25 freezing bags (25 mL/bag) (OriGen Biomedical), frozen under a standard controlled-rate protocol of ~1°C min^{-1} to reach −80°C, and then stored at liquid nitrogen (LN_2) temperatures (−196 °C).

Cryothawing

Following storage, cells were rapidly thawed both in a 37 °C water bath and the SmartThaw device until the ice dissipated and the samples were cold (~0-4°C). A one-step dilution (1:12) in growth medium was performed, and then 100 µl of the cell suspension was pipetted into individual wells of a 96-well tissue culture plate (CELLTREAT) and cultured in standard conditions (37 °C, 5% CO_2/95% air) for recovery and assessment. Growth medium was replenished at 24-hour intervals for cell survival studies.

Cell Viability Assay

To assess cell viability, the metabolic activity assay alamarBlue™ (Thermo Fisher Scientific) was utilized. Cell culture medium was aspirated from the 96-well plates and 100 µl of the working alamarBlue solution (1:20 dilution in Hank's balanced salt solution [HBSS])was applied to each well. Samples were then incubated for 60 min (±1 min) at 37 °C in the dark. The fluorescence levels were analyzed using a SPECTRAFluor Plus (Tecan) plate reader. Relative fluorescence units were converted to a percentage, compared to normothermic controls set at 100%, and the data was graphed using Microsoft Excel. Viability measurements were taken at 1, 2, and 3 days post-recovery.

Thermal Profile Assessment

During the thawing interval, the evaluation of sample temperature profiles (internal and external) was conducted using an Omega type-T thermocouple (TC) and recorded in real-time. Thermal data was collected using the Omega OMB-DAQSCAN-2000 system with the OMB-DBK90 expansion module. The system was set to an acquisition rate of 1 scan/sec to enable real-time data acquisition.

Internal Temperature Monitoring: Internal sample temperatures were recorded using a TC placed into the center of the sample volume via a rigid 19-gauge needle inserted through a luer access port in the OriGen freezing bags, or through a hole drilled in the cryovial lid. TCs were glued into place in the various containers to prevent seepage of LN_2 into the sample.

External Temperature Monitoring: For samples thawed using SmartThaw, the sample temperature was also assessed with the integrated thermo-pad (for freezing bags) or thermo-boot (for vials) to facilitate non-invasive external sample temperature monitoring.

Water Bath Thawing: Prior to removal from LN_2, internal sample TCs were connected to the Omega system and thermal recording was started after placing the samples into the 37 °C water bath. The samples were gently hand-agitated throughout the thawing process.

SmartThaw Thawing: The internal TC was connected to the Omega system prior to LN_2 sample removal, and the recording was started. In separate thaw runs, an integrated thermo-pad was positioned in the center of the sample bag, and a thermo-boot was attached to the cryovial before being placed onto the center of the thawing pillow. The SmartThaw lid was closed and the thaw program was initiated.

Post-Thaw: Each sample thawing process was concluded once the material had melted completely and attained a temperature of ~0 to 4 °C. The resulting thermal data was exported and graphically displayed using Excel software.

Data Analysis: Viability experiments were repeated a minimum of three times with an intra-experiment repeat of seven replicates ($N\geq3$, or $n\geq21$ per condition). Thermal profile evaluations were conducted on a minimum of three separate experiments for each container type. Standard errors were calculated for viability values, and single-factor analysis of variance (ANOVA) was utilized to determine statistical significance.

Results and Discussion

SmartThaw Overview and Operation

The temperature-controlled, bench-top thawing device contains a series of thermal heating pillows within the core of the unit (**Figure 1**), and it can be set to a range between ambient temperature (nominally 18°C) and 90°C by an electronic, computer-based user interface. A thaw-pillow operating temperature of 40°C (for freezing bags), and 55°C (for cryovials) was utilized in this study. Once the desired temperature is set, the unit warms up by activating a thermoelectric heater located beneath the pillows. When the desired temperature is reached, the system is ready for use. To thaw the samples, the lid of the unit is opened to expose the lower thermal pillow of the thaw chamber upon which the frozen sample is placed. The thawing surface is a flexible, compliant cushion that conforms to the sample, and thereby increases the surface contact and heat transfer efficiency, regardless of the sample shape. Then an integrated, external sample thermocouple is positioned in the center of the sample, and the unit's lid is closed. A second thawing pillow is integrated into the lid so that when it is closed, the sample is surrounded by two conforming pillows within the thaw chamber to further improve sample warming. The fully sealed heating pillows are filled with a proprietary biocompatible liquid that increases heat transfer, capacity, stability, and sample compliance. This design prevents samples from coming into contact with the liquid medium, and thereby reduces the potential of contamination. The SmartThaw thaw chamber is positioned on a motion table for rocking oscillation of the sample and pillows to maximize even heat transfer throughout the process.

A timer integrated into the operating program automatically tracks thaw times, which typically range from 2–5 minutes depending on sample volume and container shape. Throughout the process, the sample temperature is monitored via the integrated, surface thermocouple. Following an initial 30-second equilibration period during the thaw, sample temperatures are displayed in real-time throughout the remainder of the thaw interval. This delay is a result of the thermocouple adjusting to the sample temperature and does not affect sample thawing. The temperature data can be recorded and saved using the PC-based user interface (AzeoTech DaqFactory data acquisition module) in a CSV file. If desired, the data can be exported to Excel for downstream analysis. With 10 seconds remaining in the programmed thaw interval, an alarm sounds to alert the user to remove the sample. These features provide for a platform which meets the diverse needs, environments, and compatibility of any type of sample or container utilized for sample freezing and holding.

Thaw Profile Comparison

To assess the thermal profile of the samples during thawing, samples cryopreserved in 2 mL cryovials (1 mL sample volume) or CS 25 freezing bags (25 mL sample volume) were thawed with both the standard 37°C water bath protocol and SmartThaw. Samples were frozen with a TC inserted into the center of the sample volume to allow for direct assessment of sample temperature during thawing. When samples frozen in CS 25 freezing

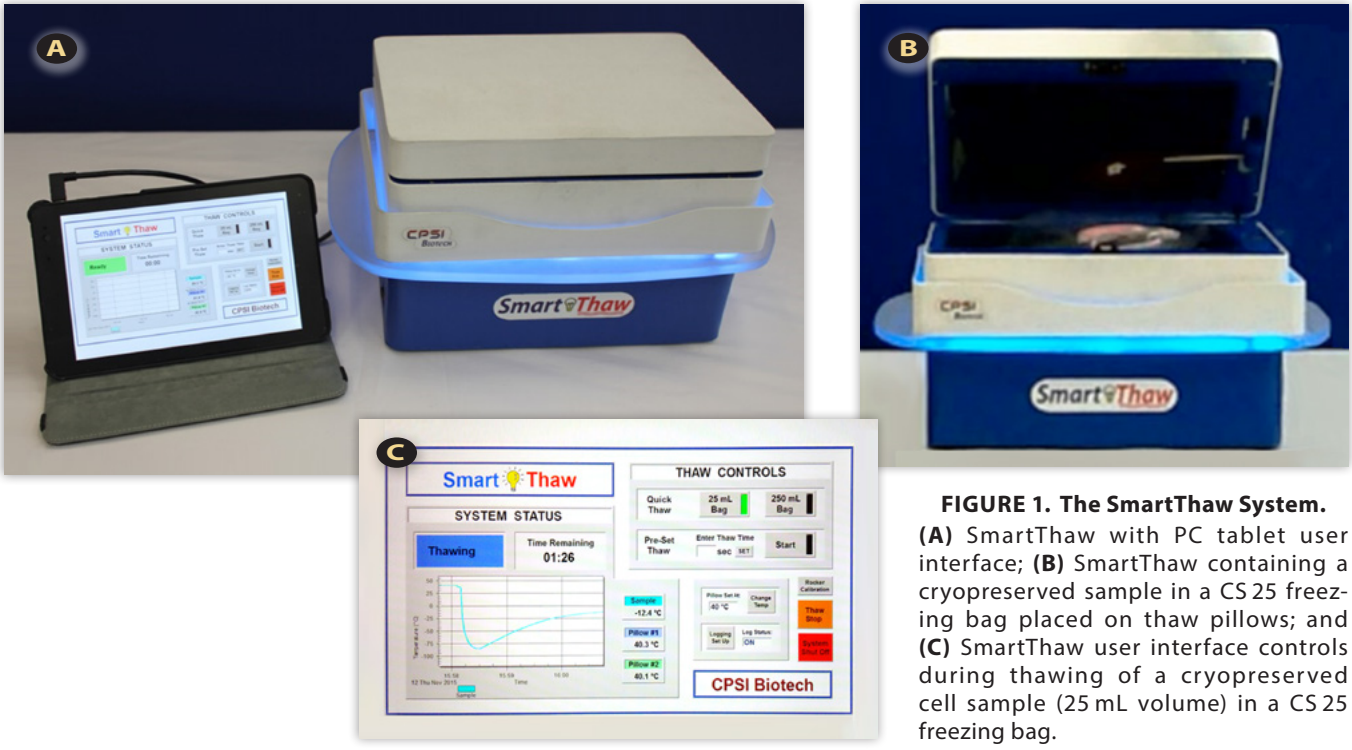

FIGURE 1. The SmartThaw System. **(A)** SmartThaw with PC tablet user interface; **(B)** SmartThaw containing a cryopreserved sample in a CS 25 freezing bag placed on thaw pillows; and **(C)** SmartThaw user interface controls during thawing of a cryopreserved cell sample (25 mL volume) in a CS 25 freezing bag.

Emerging Targeted Therapies in Cancer and Autoimmunity

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The four-day event will bring together basic scientists, medicinal chemists, pharmacologists, biotechnologists, clinical investigators, and other allied professionals from around the world interested in the fields of disease pathogenesis and new targeted therapies developed for cancer and autoimmune disorders. The meeting will be an excellent opportunity to expose the translational nature of modern biomedical and pharmaceutical research. The official language of the workshop is English.

Main Workshop Topics:

Cancer

- Molecular oncology
- Tumor immunology
- Cancer immunotherapy
- Clinical trials

Autoimmunity

- Mechanisms of autoimmune-related diseases
- Regulatory T cells and autoimmunity
- Pharmacological strategies for treatment

Organized by the Center for Genetic Engineering and Biotechnology (CIGB) Biomedical Research Direction

Click here to download the program. More information on this meeting, including transportion and accommodations, and other CIGB international meetings on biomedical research can be found at http://biomed.cigb.edu.cu/.

Center for Genetic Engineering and Biotechnology
31 Ave e/ 158 and 190
Cubanacan, Playa, Havana 10600, Cuba
Email: biomed@cigb.edu.cu, Phone: (53-7) 208 7465

bags were removed from LN_2 and placed into the water bath or SmartThaw, rapid warming was observed (**Figure 2A**). With the SmartThaw, CS 25 samples warmed from –196°C to ~–5°C in 2 min/30 sec and proceeded to warm through the latent heat of fusion stage (complete melting) within 3 mins. This was similar to water bath-thawed CS 25 samples which warmed to ~–5°C in 3 mins and completely melted by 3 min/30 sec (**Figure 2A**).

Comparable studies were conducted using standard 2 mL cryovials (1 mL sample volume). The cryovial thaw profiles obtained with the water bath and SmartThaw were nearly identical with samples rapidly warming to the latent heat of fusion transition plateau in ~2 mins and completely thawing by ~3 mins (**Figure 2B**). These studies revealed no significant difference between the overall thawing rates and times with samples frozen in freezing bags or cryovials when comparing SmartThaw to standard 37°C water bath thawing protocols. However, with SmartThaw, the samples required no manual manipulation during thawing, and the automated rocking oscillation optimized temperature distribution uniformity. SmartThaw also maintained a cleaner, dryer environment, thereby reducing the risk of contamination with less post-thaw cleanup and processing.

Internal vs. External Sample Temperature Monitoring

One aspect of sample processing which is gaining interest is the ability to actively monitor the thermal profile of a sample during the freezing and thawing intervals. Currently, there is no standardized mechanism to actively measure sample temperature during thawing, short of placing a TC directly into a sample prior to freezing, which is not practical. For the SmartThaw, we developed a series of external thermo-sensors that are compatible with various freezing bags and cryovials. After testing a number of design iterations, external thermo-pad (for freezing bags) and thermo-boot (for vials) devices were developed. The thermo-pad could be placed on the outer surface of a freezing bag during the thaw interval to monitor the sample temperature without the need for an internal TC. To assess

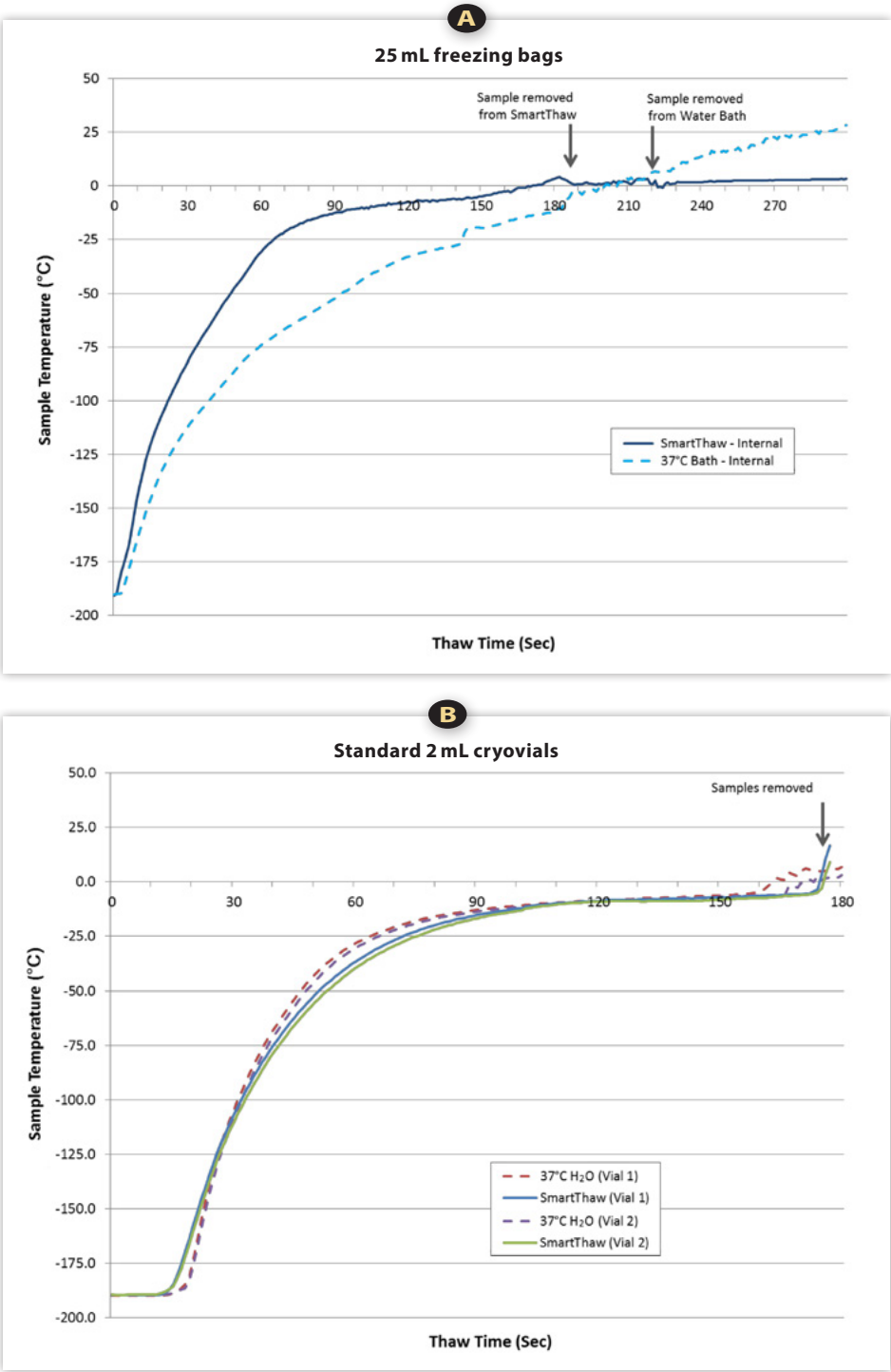


FIGURE 2. Comparison of internal thermal profiles of samples thawed using SmartThaw and a 37°C water bath. Samples were cryopreserved in: **(A)** 25 mL freezing bags; or **(B)** standard 2 mL cryovials; and thawed using SmartThaw (solid line) or standard 37°C water bath (dotted line). The data illustrate similar thawing profiles attained using the two methods.

the accuracy of the external thermo-pad monitoring approach, a series of studies were conducted using CS 25, CS 250, and CS 500 freezing bags. They were filled to the manufacturer's recommended volumes of 25 mL, 70 mL, and 100 mL respectively. As with the thermal profile studies described earlier, a TC was placed into each sample prior to freezing.

Following an initial ~30 second adjustment period, the temperatures recorded for CS 25 samples revealed that the temperatures measured with the internal and external sensors were similar (**Figure 3A**). Importantly, within the critical ice melting window (latent heat of fusion temperature range), the temperatures recorded internally and externally were not statistically different. This illustrates that the integrated, thermo-pad external sample monitoring system of SmartThaw provided an effective means of monitoring sample temperature during thawing, particularly during the critical transition stage where ice crystals fully dissipate, thereby reducing the risk of sample under-thawing or overheating. Further, it was noted that the initial ~30 sec adjustment period, when the external thermo-pad adjusts to the sample temperature, did not affect the sample thaw profile.

Using external temperature monitoring of the CS 25 samples, studies were expanded to the larger volume CS 250 (70 mL/bag) and CS 500 (100 mL/bag) freezing bags. As in the CS 25 studies, a thermocouple was placed into the sample prior to freezing to compare internal sample temperature with external recordings. The thermo-pad temperature profiles obtained for the CS 250 and CS 500 samples were highly similar to the internal temperature measurements (**Figures 3B** and **C**). As with the CS 25 samples, an adjustment

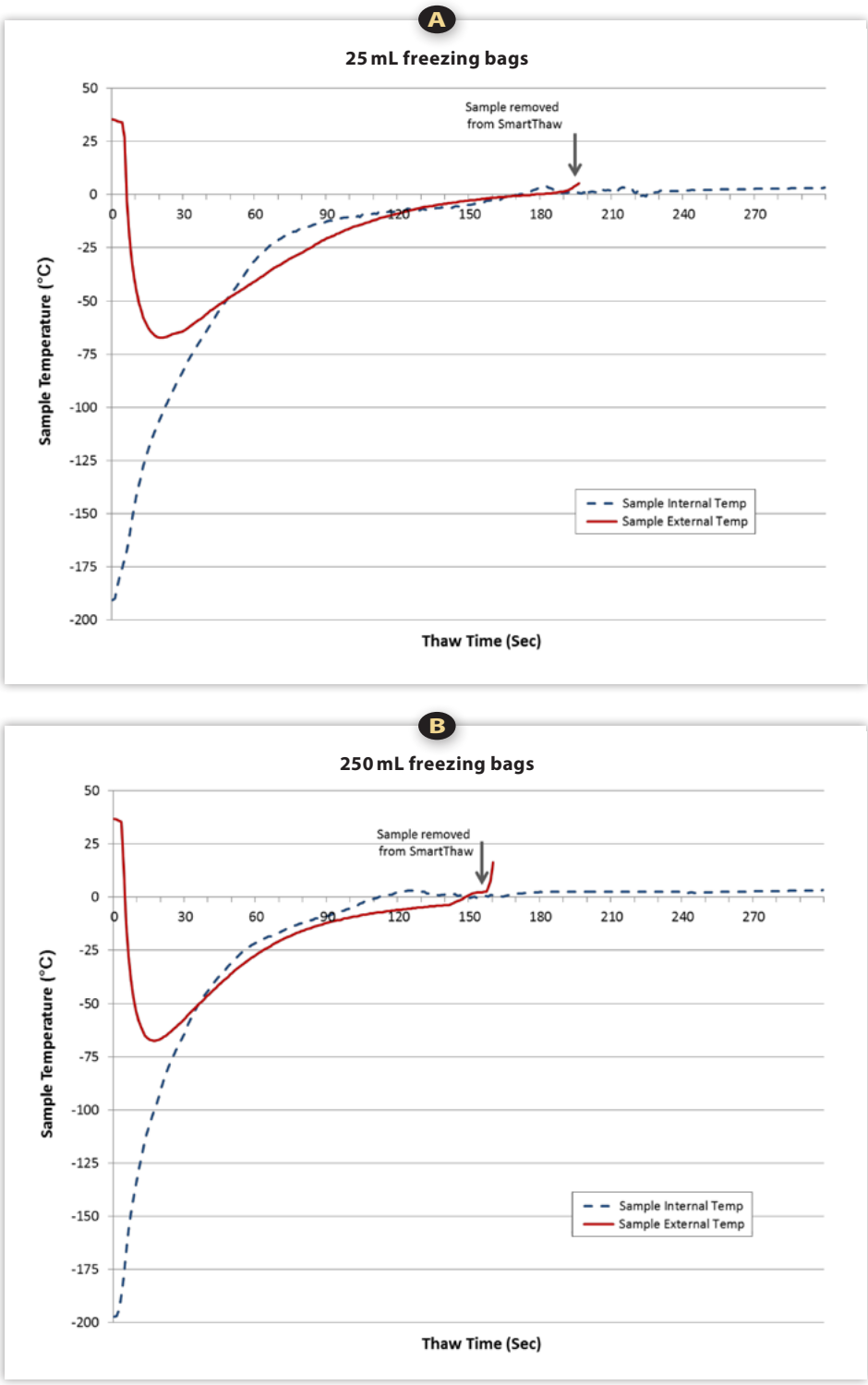
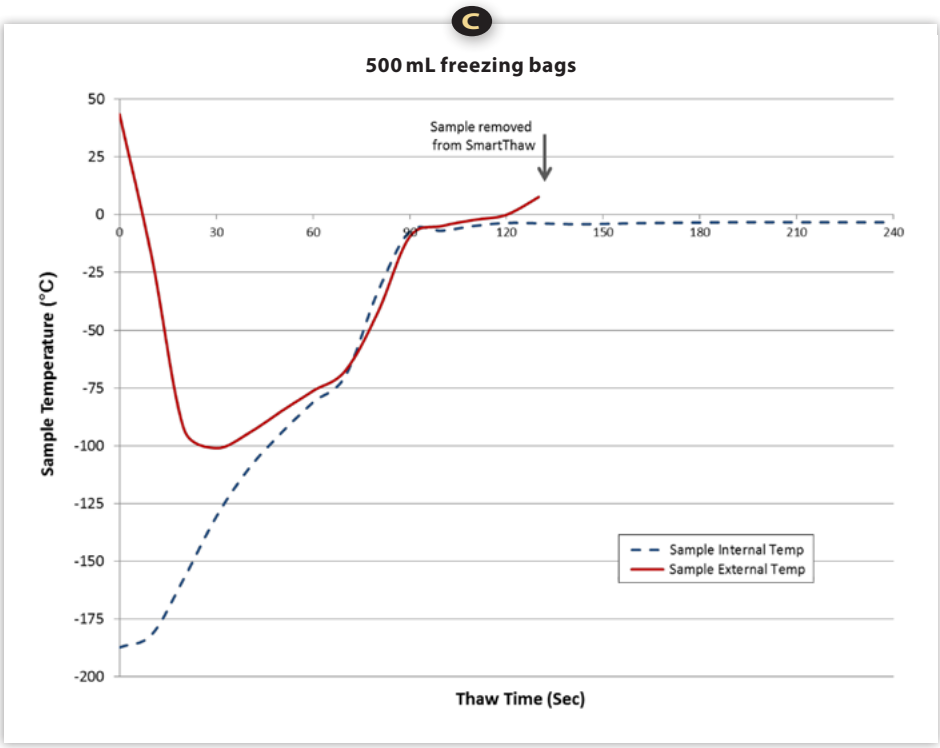


FIGURE 3. Comparison of sample temperature monitoring during thawing using internal vs. external monitoring. Samples were cryopreserved in freezing bags: **(A)** 25 mL size (25 mL volume); **(B)** 250 mL size (70 mL volume); and **(C)** 500 mL size (100 mL volume) (shown on the following page); and then thawed using SmartThaw. The sample temperature was monitored using an internal TC and an external thermo-pad. Following a 30-sec equilibration period at the beginning of the thaw interval, internal and external temperatures were found to be similar between the two monitoring methods. This was particularly so in the critical latent heat of fusion window (−5°C to 0°C) where ice crystals fully dissipate. Sample temperature monitoring provides a history of the sample thermal excursion and prevents overheating, which is common in water bath protocols.



period was seen during the initial ~30 seconds of thawing, but once thermal equilibrium between the sample and thermo-pad was achieved, internal and external sample temperature profile recordings tracked similarly with one another, allowing for monitoring and recording of the thaw process. Interestingly, complete thawing of the CS 250 (2 min/30 sec) and CS 500 (2 min) samples took place in less time than the smaller CS 25 (3 min). This decrease is believed to have been caused by the larger size/increased surface area of the CS 250 and CS 500 freezing bags.

Post-Thaw Cell Survival

Using thawing profiles achieved with SmartThaw that were similar to those of a 37°C water bath, we conducted a series of post-thaw viability studies to compare the samples thawed with the two systems. The PC-3 human prostate cancer cells and primary hMSCs were selected as they ranged from easy (PC-3) to difficult (hMSC) to cryopreserve and thaw.

Both PC-3 and hMSC samples were cryopreserved using the standard controlled rate (–1°C/min) freezing method described earlier. After storage in LN_2, they were thawed using both the water bath and SmartThaw protocols. In addition to comparing the thawing methods, the various CP solutions (culture media, Viaspan, and Unisol) were utilized to assess the compatibility of SmartThaw for these media formulations. In these studies, sample viability was assessed at 24 hours post-thaw to allow for manifestation of cryopreservation-induced delayed-onset cell

death (CIDOCD), which occurs several hours post-thaw and significantly impacts (reduces) sample viability.[24–26] It is recommended that true cell viability be assessed well beyond a few hours post-thaw due to the inevitability of CIDOCD.[27, 28]

With the PC-3 samples cryopreserved in media plus 10% DMSO and then thawed with the water bath and SmartThaw protocols, no significant difference was revealed in sample viability at 24 hours post-thaw (75.1% [±5.4] vs. 72.3% [±3.9], $P>0.1$) (**Figure 4A**). Similar results were obtained with samples cryopreserved in Viaspan and Unisol plus 15% DMSO. The 24-hour post-thaw survival measurements yielded by the water bath and SmartThaw methods were within 2% of each other for the three solutions (**Figure 4A**). Interestingly, when a reduced DMSO concentration (5% vs. 15%) was utilized in conjunction with the Unisol carrier media, a marked reduction in post-thaw viability was noted in the water bath-thawed samples (41.5% [±4.7] vs. 66.5% [±4.9], $P>0.1$). However, with the SmartThaw samples, overall viability was equivalent to that obtained with higher DMSO levels (64.2% [±4.7] vs. 66.5% [±3.5], $P>0.1$). These data point to the potential of reducing the cryoprotectant agent (CPA) concentration while maintaining elevated survival when utilizing the SmartThaw in combination with the Unisol carrier medium.

Studies conducted with hMSCs yielded similar results to those of the PC-3 experiments. Specifically, hMSCs thawed with a water bath yielded a 60.4% (±10.0) survival vs. 58.8% (±6.6), $P>0.1$, with SmartThaw (**Figure 4B**), while hMSC samples cryopreserved in Viaspan plus 15% DMSO yielded post-thaw viabilities of 64.9% (±9.9) vs. 62.4% (±11.1), $P>0.1$, respectively. Cryopreservation of hMSCs in Unisol plus 15% DMSO resulted in an overall improvement in post-thaw survival as compared to the media and Viaspan samples. However, no significant thawing difference was found between the water bath and SmartThaw methods (75.1% [±8.5] vs. 68.7% [±5.0], $P>0.1$). Overall, the viability studies using the PC-3 and hMSC cell models revealed comparable post-thaw survival between SmartThaw and traditional water bath protocols.

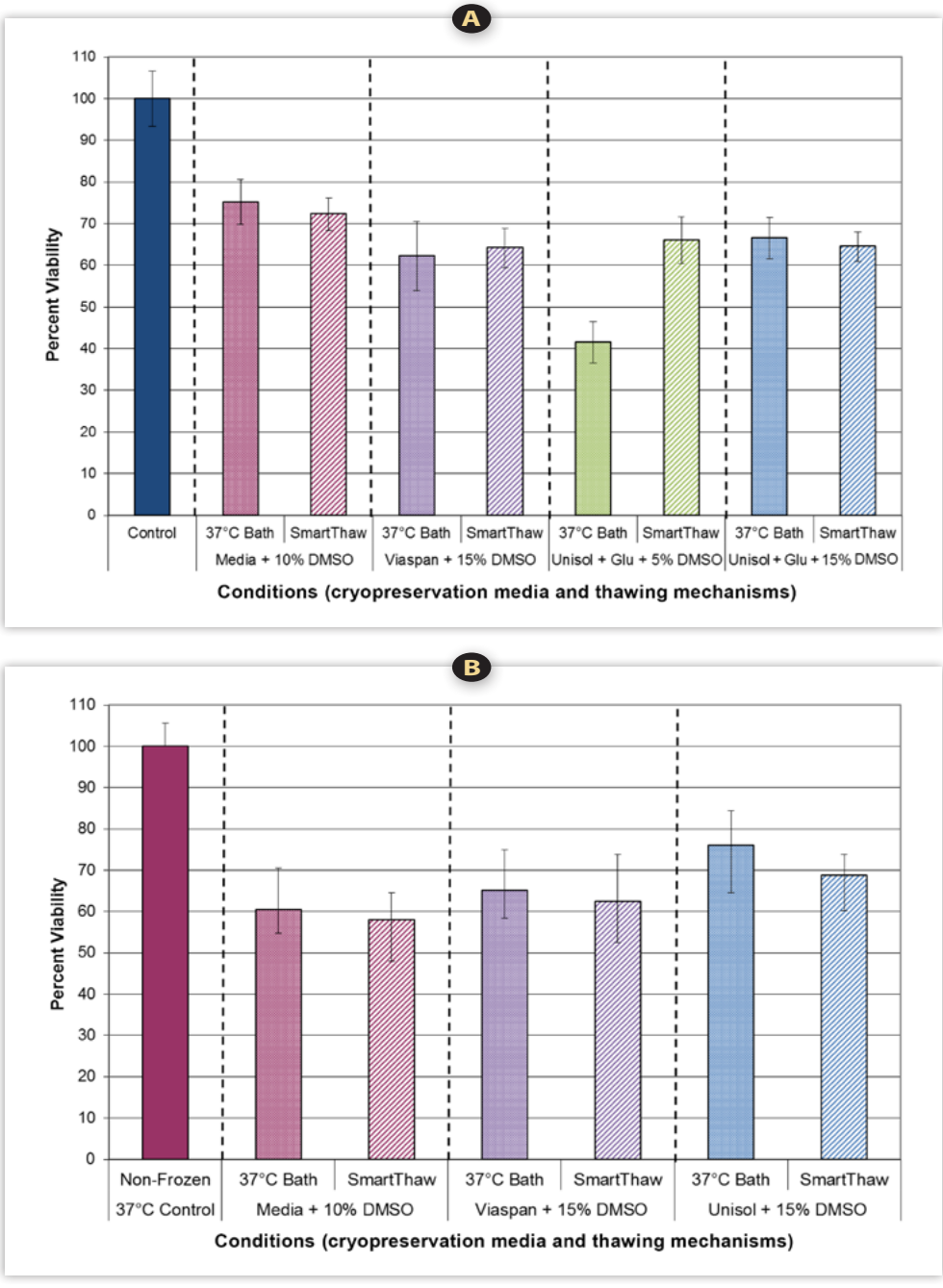


FIGURE 4. Assessment of 24-hour post-thaw cell survival. Cell samples: **(A)** PC-3 and **(B)** hMSC were cryopreserved in various carrier media formulations supplemented with DMSO and then thawed using both the SmartThaw and water bath protocols. Assessment of sample viability revealed similar post-thaw survival between the two methods. However, SmartThaw provided clean, dry, hands-free, automated thawing.

Cell Recovery and Repopulation

In addition to impacting initial post-thaw survival, CIDOCD has been shown to impact cell system recovery and repopulation following thawing.[26,32] As such, we examined sample recovery over a 3-day post-thaw interval, and PC-3 samples revealed an overall positive increase in cell populations (**Figure 5A**). While there was an overall trend in cell repopulation, the rate varied depending on the CPA utilized. In the media plus 10% DMSO formulation, samples thawed with SmartThaw recovered at a rate similar to those of water bath-thawed samples (**Figure 5A** Δ samples). PC-3 cells cryopreserved in Unisol plus 15% DMSO and thawed using SmartThaw showed a significant improvement in repopulation by day 3 (209% [±3.5]) as compared to water bath-thawed samples (173% [±4.9], $P<0.005$), despite similar day 1 survival. As with the media plus 10% DMSO samples, the PC-3 cells cryopreserved in either Viaspan plus 15% DMSO or Unisol plus 5% DMSO recovered at a similar rate when thawed using SmartThaw as compared to the results in a water bath. Final recovery levels were based on their respective starting points (**Figure 5A** ◇ and ✳). Similar trends in recovery were also noted in hMSC experiments (**Figure 5B**). hMSCs in media plus 10% DMSO or Viaspan plus 15% DMSO and thawed using either SmartThaw or the water bath yielded nearly identical recovery trends (**Figure 5B** Δ and ◇, respectively). However, as with the PC-3 cells and hMSCs cryopreserved in Unisol plus 15% DMSO, the samples thawed with SmartThaw resulted in a significant improvement in post-thaw recovery when compared to its water bath-thawed counterpart following 3 days of recovery (206.1% [±19] vs. 163.1% [±21] respectively, $P<0.005$). In these studies, it was found that hMSCs cryopreserved in Unisol plus 15% DMSO and thawed using SmartThaw resulted in the best overall survival when compared to all other conditions assessed. Samples thawed using SmartThaw were found to have a similar, or improved, repopulation over the 3-day post-thaw recovery interval experienced with the water bath-thawed samples.

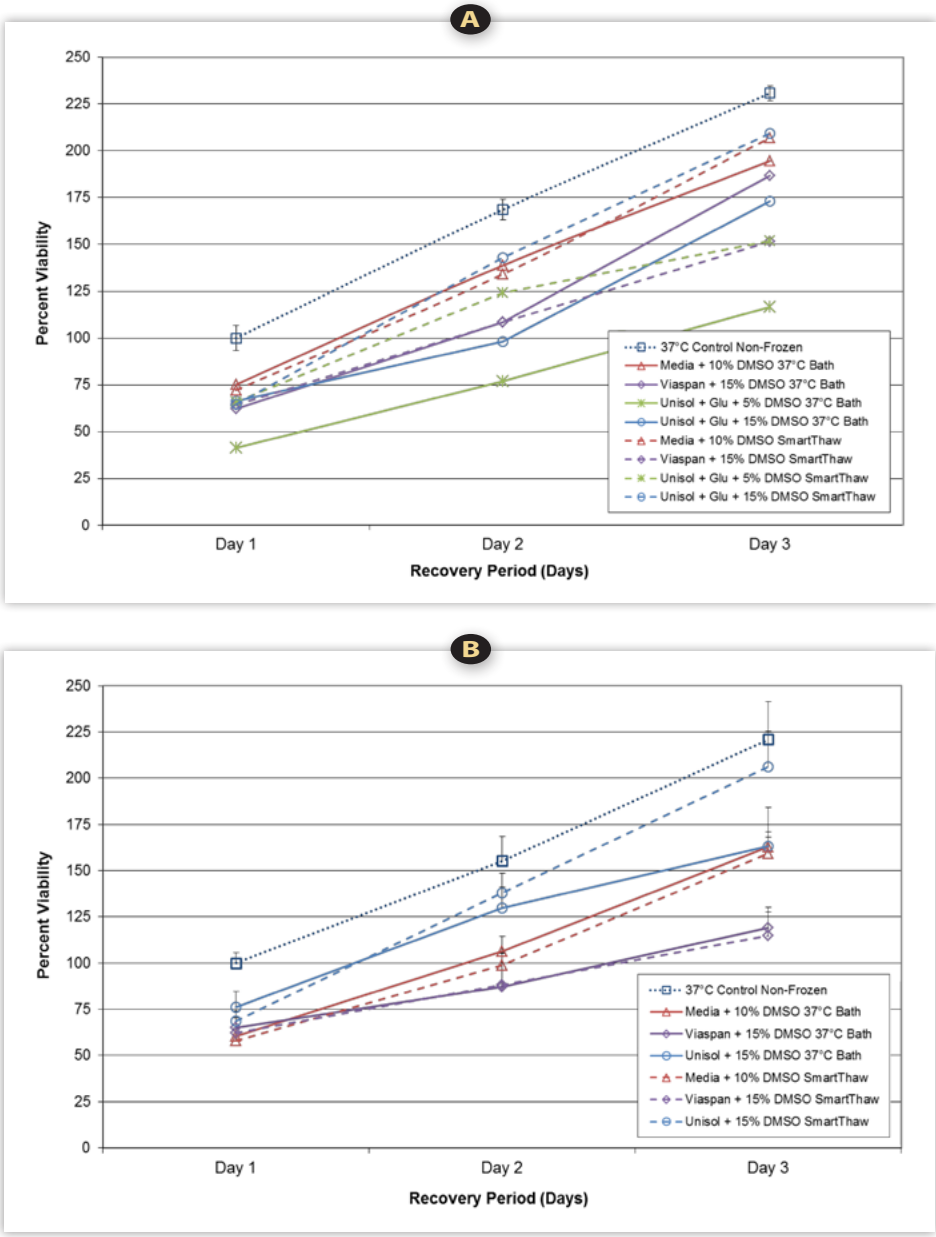


FIGURE 5. SmartThaw vs. water bath comparison of post-thaw sample recovery. Cell samples: **(A)** PC-3 and **(B)** hMSC were cryopreserved in various CPA formulations supplemented with DMSO and then thawed using both protocols. Over a 3-day post-thaw interval, sample viability and repopulation was found to be similar to, or improved, in the repopulation samples thawed using SmartThaw as compared to those thawed using a standard 37°C water bath protocol.

Summary

In this study, we evaluated sample warming rate, viability, and recovery following sample thawing with SmartThaw, and compared the outcomes to those obtained with a standard 37°C water bath protocol. The SmartThaw system, with the thermal pillows set to 40°C,

was able to generate thawing rates of ~60°C/min, which was similar to that of the standard water bath protocol. Additional studies also demonstrated the ability to adjust the overall thaw rate using SmartThaw by increasing or decreasing the thaw pillow temperature prior to sample thawing (data not shown). This unique feature allows a user to customize the thaw rate (time) based on individual parameters to optimize sample conditions. In addition to standard cryovial and 25 mL freezing bags, studies conducted with 250, 500, and 1000 mL freezing bags have illustrated enhanced thawing outcomes as compared to those obtained with water baths. This was achieved with SmartThaw while providing for a clean, controlled, and documentable process, which is not possible with a water bath thaw protocol. Assessment of cell viability following thawing using prostate cancer and human stem cells revealed similar survival results using SmartThaw. This pattern was observed across a variety of carrier media and cryoprotective agent concentrations. Follow-up assessment of the populations over a 3-day post-thaw recovery demonstrated that the surviving cells were also able to divide in culture at a similar, or in some cases, an improved rate of recovery using SmartThaw. These data demonstrate the highly effective nature of SmartThaw when compared to a standard warm water bath thaw method, while simultaneously mitigating several potential risk factors.

Conclusion

The data show that SmartThaw was capable of thawing cells in vials or cell therapy bags and demonstrated its potential utility in basic research, cell bioprocessing, and clinical settings. Unlike a water bath, SmartThaw also reduced the risk of contamination and minimized operator-dependent variability. The integrated external thermo-pad monitoring and logging of sample temperature during the thaw process was found to be of added benefit. The combination of providing consistent, hands-free, documentable thawing of a variety of sample containers, volumes, and manufacturer brands (various sized freezing bags, vials, and ampoules, among others) in a clean, dry environment while providing similar or improved cell survival and recovery suggests that SmartThaw provides an important improvement in cryopreservation processes. Given the diversity of industries that rely on processing and banking biologics for downstream utilization, SmartThaw may provide an enabling, user friendly thawing device for use in areas including biopharmaceuticals (drug discovery), vaccine development, stem cell research, cell and gene therapy, toxicological testing, cosmetics, biodefense, diagnostic healthcare, environmental monitoring, and basic research science.

Acknowledgment

The authors would like to thank Joshua Smith, BS ME, Kimberly Santucci, BS, and Anthony Robilotto, MS, for their technical assistance in the SmartThaw construction and thermal profile data collection. This study was funded in part by NIH Grant #1R43RR032140-01 and by CPSI Biotech.

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About the Authors

John M. Baust, PhD*[1,2], William L. Corwin, PhD[1,2], Kristi K. Snyder, PhD[1,2], John G. Baust, PhD[2,3], and Robert G. Van Buskirk, PhD[1,2,3]

1. CPSI Biotech, 2 Court St., Owego, NY 13827 USA
2. Institute of Biomedical Technology, Binghamton University, Binghamton, NY 13902 USA
3. Department of Biological Sciences, Binghamton University, Binghamton, NY 13902 USA

Corresponding Author:
CPSI Biotech, 2 Court St., Owego, NY 13827 USA
Email: jmbaust@cpsibiotech.com | Phone: 607-687-8701

Dr. John M. Baust presented portions of this article at the **ISBioTech 3rd Fall Meeting** held in Rosslyn, Virginia USA, September 28–30, 2015. Presentation title: *Continued Development of Novel Freezing and Thawing Devices for Improving Cell Cryopreservation*.