Next Generation Technology, Procedures, and Products Facilitate Biopreservation Best Practices for Cellular Therapies.

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ABSTRACT

The quality of procedures and products used for preparing, transporting and storing cells at cryogenic temperatures have a direct impact on post-thaw viability and functionality, as well as the consistency and reliability of biological agents from a Quality perspective. Two methods of preparing, transporting and storing living cells were compared to evaluate how the implementation of best practices impact post-thaw cell viability and functionality. Jurkat T cells were cryopreserved in either CryoStor® CS5 and shipped in the CRYO evo™ SMART Shipper under continuous environmental monitoring, which represented an optimized cold chain (OCC), or cryopreservation media composed of 95% FBS + 5% DMSO (95/5%) and shipped in an unmonitored expanded polystyrene box (EPS), which represented Current Practice (CP). Samples were transported from Bothell, WA (BioLife Solutions) to Boston, MA (Brooks Life Sciences), where they were stored at -190°C in vapor phase LN_2 for 45 days. The samples were then returned to Bothell, WA from Boston, MA in identical containers as those used for initial transportation. Samples were immediately thawed upon arrival, and were assessed for viability and metabolic function post-thaw, and at 24 and 48 hours post-thaw. Non-shipped cryopreserved samples were used as a control condition (Ctrl). The results suggested significant loss of viability due to shipment in the CP group, while no significant change in viability was observed in the OCC group. Further analysis of cell recovery using a functional assay suggested a 30% loss of metabolic activity in the CP group at 48 hours post-thaw compared to the non-shipped control. On the other hand, recovery of the OCC group was statistically identical to the non-shipped control. While the identification of the root cause of T cell dysfunction in the CP group was not within the scope of this study, likely causes include sub-standard preparation, improper cryoprotectant exposure and poorly controlled shipping conditions. The outcome of this study supports the incorporation of Best Practices into cell preparation, storage and transportation to ensure consistency and control of the cold chain, and to improve the functionality of biologic products.

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INTRODUCTION

Cold Chain is an industry term representing all the aspects of handling and distribution of cellular products, which include preparation, transportation and storage, usually in a hypothermic or cryopreserved state. The quality of procedures and products used in each of these steps have direct impact on the return-to-function of the cellular products post-thaw. Inherently, sub-optimal preparation, handling and storage may subject cells to excessive stresses that negatively impact the biological function of manufactured cell products. Furthermore, inadequate transport packaging and negligent shipping practices can cause transient warming events and variability in product temperatures throughout shipping and distribution. As a result, the viability, recovery and functionality of therapeutic doses could be negatively affected and contribute to poor clinical outcomes. Therefore, it is necessary to define and implement Optimized Cold Chain practices to ensure the highest quality commercial cellular products.

Cryopreservation is generally the mechanism for long-term storage, and one preferred method for transportation of cell doses due to a number of biological and logistical reasons, which include but are not limited to, patient scheduling concerns, clinical operating hours, and laboratory testing. Currently, most common cryopreservation protocols include the use of serum/protein-containing isotonic media supplemented with dimethyl sulfoxide (DMSO) at various concentrations, passive freezing to -80°C at -1°C/min, and then transfer to liquid nitrogen vapor storage (LN_2). For commercialized cellular products, the use of serum and other components directly derived from animals is discouraged from manufacturing and processing to mitigate source variability and to minimize the risk of xenopathic disease transmission to patients[1]. Consequently, well-defined GMP manufactured media for cell culture and cryopreservation are preferred and are in increasing demand in the developing field of cellular therapies. In order to transport cellular products from the manufacturing site to the patient bedside, current practice may rely on the transfer of vials or bags from liquid nitrogen to an expanded polystyrene (EPS) shipping container filled with approximately 10-20 kg of dry ice to ensure temperature stability during shipment, or LN_2 dry shippers. Cell products are then typically transported using traditional freight carriers, which can be subject to logistical delay, improper handling, and undue environmental exposure with minimal documentation. To avoid the unanticipated events associated with traditional freight carriers, some cell therapy groups employ expensive courier or "white glove" services to ensure monitored and on-time patient delivery[2].

The current practice for the cryopreservation and transportation of cellular products includes a combination of suboptimal freezing in protein/serum containing, saline-like isotonic-based cryopreservation media and shipping in Styrofoam boxes at dry ice temperatures, or LN_2 dry shippers, with minimal analytic information during shipment. The objective of this study was to compare the performance of focused aspects of the current practices (CP) in preserving viability of the precious cell dose to that of an optimized cold chain (OCC), which couples biopreservation Best Practices (i.e. the use of serum-free, intracellular-like cryopreservation media manufactured under GMP) with an advanced SMART shipper with integrated continuous environmental recording. This first

study was focused on the method of dry ice shipments, and not LN_2 dry shippers. The outcome of this focused study recommends Best Practices for procedures and products to ensure consistency, visibility, and documented control of the cold chain.

MATERIALS AND METHODS

Cell Culture

The Jurkat human acute T cell leukemia cell line (ATCC, Manassas, VA) was cultured in complete growth medium (CGM) consisting of RPMI 1640 media (Lonza, Walkersville, MD) supplemented with 10% v/v fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO) and maintained in a 5% CO2 incubator at 37°C. In both culture and experimental conditions, cell density was maintained between 0.5-2×10^6/mL to facilitate an exponential growth rate. Immediately prior to freezing, cell viability was determined by membrane integrity via manual assessment of Trypan Blue exclusion using a hemocytometer.

Cryopreservation Procedures

For the cryopreservation of all test samples, a volume of cell culture media containing 5x10^6 cells was pelleted in a 15 ml conical tube via centrifugation at 250g for 5 min. Following centrifugation, the media was decanted and the cells were resuspended in 1 ml of cryomedia and transferred to pre-chilled FluidX® 2 mL external threaded cryovials. Two different types of cryomedia were used for this study: (1) "Current Practice" cryomedia was prepared by mixing 0.2µm syringe-filtered FBS with dimethyl sulfoxide (DMSO, BloodStor® 100, BioLife Solutions, Bothell, WA) at 95:5 volume ratio, and (2) for "Optimized Cold Chain" cryomedia, CryoStor® CS5 (BioLife Solutions, Bothell, WA) was used. The cryovials were placed into a pre-chilled isopropyl alcohol passive freezing container and stored at 2-8°C for 10 min to facilitate temperature equilibration. Following equilibration, the freezing container was transferred to a -80°C freezer and manual ice nucleation was performed 20 min after transfer. After 3 hours, the cryovials were submerged in LN_2 for a minimum of 18-24 hours prior to shipment.

Across the broad spectrum of cell types, CryoStor is more effective in reducing post-preservation necrosis and apoptosis as compared to commercial and home-brew isotonic and extracellular formulations. This enables greatly improved post-thaw cell yield, viability, and recovery.

Shipping, Handling and Storage

For the purpose of this study, cells were cryopreserved and shipped using two different methods: (1) Best Biopreservation Practices and Optimized Cold Chain (OCC), which consisted of combination of CryoStor® CS5 for cryomedia and the CRYO evo™ SMART Shipper with dry ice for transport, and (2) Current Practices (CP), consisting of a mixture of serum and DMSO (95% and 5% v/v, respectively) and extruded polystyrene (EPS) boxes filled with dry ice. **Fig. 1** represents the workflow of sample preparation and shipment under the two different methods. The cryovials containing the prepared samples were removed from LN_2 storage and transferred immediately into either the CRYO evo™ SMART Shipper preloaded with 5kg of dry ice or into an EPS box shipping container preloaded with 2.5kg of dry ice. The EPS box was then loaded with payload and an additional 2.5kg of dry ice was added to the box to match the total dry ice content of the CRYO evo™ SMART Shipper. This methodology ensured cryovials were completely surrounded in dry ice pellets prior to shipment. Cryovials containing Jurkat cells frozen in CS5 were transported in CRYO evo™ SMART Shippers (OCC), whereas cryovials containing Jurkat cells frozen in 95/5% reference cryomedia were transported in EPS containers (CP). Shipments were shipped between BioLife Solutions and Brooks Life Sciences via UPS 2-day air freight.

Upon receipt at the Brooks facility, the OCC and CP parcels were weighed, unpacked and the cryovials immediately moved into their respective Brooks CryoPod™ carriers (<-150°C). The two CryoPods were then carried to the Brooks

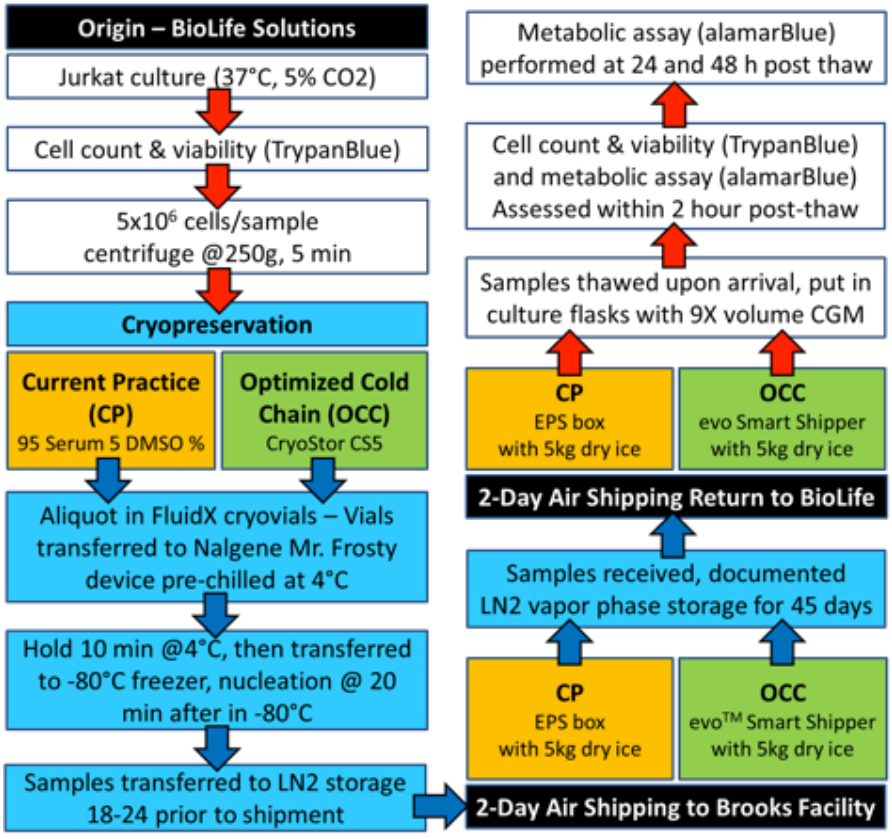

Figure 1. Workflow diagram of sample cryopreservation and shipment to Brooks facility from BioLife Solutions and back.

BioStore™ III Cryo automated LN_2 storage system (B3Cryo) in a separate building. The cryovial barcodes were scanned and registered into the B3Cryo system and permissions set so that only the owner could retrieve them. The cryovials were stored in their respective, pre-frozen cryoboxes for 45 days at -190°C. A Brooks TempAura™ remote temperature monitoring device was already attached to the B3Cryo to provide backup temperature monitoring, recording and alarms. No alarms triggered during the 45 days, but prior to removing the cryovials an audit was run of the cryovials to ensure no access had happened and the temperature logs reviewed to ensure no warming occurred.

A similar procedure was followed for return shipment preparation.

Post-Thaw Viability and Functionality Assays

Following the return transit receipt at BioLife, cryovials were simultaneously removed from both the CRYO evo™ SMART Shipper and EPS shipper and immediately thawed in a 40°C water bath for 2.5 minutes along with the reference (non-shipped) matched controls of cells cryopreserved in CS5 and 95/5%, which had been continuously stored in LN_2 at the BioLife facility. The thawed solution containing cells was added to 9 mL of CGM and transferred to a 25cm^2 tissue culture flask. Post-thaw cell count and membrane integrity were assessed by manual determination of Trypan Blue exclusion using a hemocytometer and values normalized to pre-freeze values for each cryovial. Functional activity was assessed by the metabolic indicator alamarBlue® (AbD Serotec, Bio-Rad, CA). Briefly, approximately 1.25x10^6 cells were removed from the flask immediately post-thaw, and at 24 and 48 hours post-thaw, and pelleted in a 15 mL conical centrifuge tube at 250 g for 5 min. Following centrifugation, media was decanted and cells were resuspended in 600 µL of alamarBlue at a 1:20 dilution in Hanks Balanced Salt Solution (HBSS). Exactly 100 µL of the cell suspension in alamarBlue was added to 5 wells of a 96-well microplate and alamarBlue® fluorescence evaluated every 5 min for 1 hour using a Tecan SPECTRAFluorPlus plate reader (TECAN Austria GmbH, Austria) at 530nm/590nm excitation/emission.

Brooks BioStore™ III Cryo automated storage system

CRYO evo™ SMART Shipper

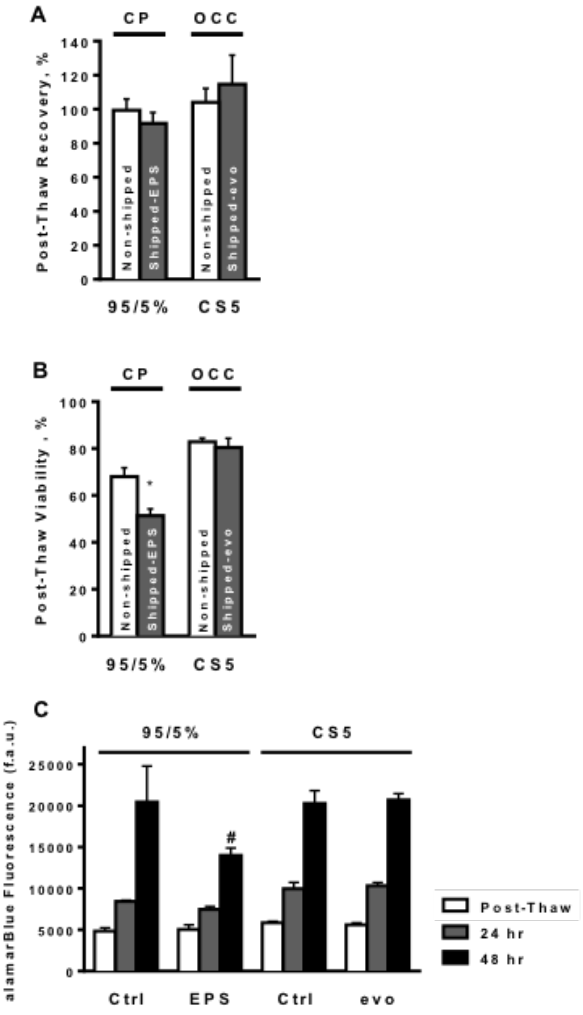

Figure 2. Optimized Cold Chain increase cell viability. (A) Post-thaw recovery and (B) viability of shipped versus non-shipped Jurkat cells. (C) Post-thaw metabolic activity of frozen Jurkat cells compared to non-shipped controls. (*p=0.0001, #p<0.0001).

Data Analysis

Where indicated, statistical analysis was conducted using 2-way ANOVA with Tukey's post-hoc comparisons and significance set at $p<0.05$ a priori. Data are presented as mean±S.E.M of 3 independent samples (n=5 replicates/sample).

RESULTS:

Cell Viability and Post-Thaw Recovery

Normalized cell count (recovery) and viability immediately post thaw are reported in **Fig. 2A** and **2B**. Recovery was calculated by normalizing cell number to the respective pre-freeze count. The results in **Fig. 2A** show equivalent cell recovery between both groups compared to corresponding non-shipped controls that approached 100% of pre-freeze values. However, cell viability experienced a decline in the CP group immediately post-thaw compared to pre-freeze viability (68.0±3.8%). In contrast to recovery, significant differences were observed in cell viability between the CP and OCC groups. Overall, Jurkat T cells frozen and shipped under current practice (CP) experienced a significant decline in cell viability versus non-frozen controls and the viability further declined after shipment to 51.4±2.9% which was statistically significant ($p=0.0001$, n=3) **(Fig. 2B)**. However, cells stored and shipped under optimized conditions (OCC) experienced elevated baseline viability (80.4±3.9%) that did not significantly decline following shipment. Direct comparison of the shipped cells in OCC and CP groups shows a meaningful decline in viability post-shipment from 82.8% to 51.4% ($p<0.0001$, n=3) respectively.

Membrane integrity is a commonly used indicator for cell viability, but does not guarantee that cells will return to function post-thaw. To evaluate how CP and OCC impact cell function post-thaw, the commercially available metabolic indicator alamarBlue was employed. In this assay, the irreversible reaction of resazurin to resorufin is proportional to aerobic respiration, and can be used as a surrogate for cell metabolism during recovery. The Jurkat T cells in the CP group experienced a significant ($p<0.0001$, n=3) delay in functional recovery by about ~30% at 48 hr post-thaw compared to their respective non-shipped control. **(Fig. 2C).** In contrast, Jurkat T cells frozen in the OCC group displayed a more rapid rise in metabolic function which was identical to non-shipped

controls at 48 hr. The ~30% decrease in functional recovery for the CP group coincides with the loss in cell viability as detected by membrane integrity. These results show that the combination of CryoStor CS5 and the CRYO evo™ SMART Shipper supported a cellular return to full metabolic functionality after 48hrs, and demonstrate the importance of employing an OCC for cryopreservation and shipping. On the other hand, cells cryopreserved in FBS/DMSO home-brew and shipped using CP methods

Shipment Monitoring & Tracking

The CRYO evo™ SMART Shipper monitored and recorded the internal temperature of the payload (Cryovials), along with time, GPS location and a record of any unanticipated open events. All of this information was automatically transmitted during shipment so that the entire sample transportation history and environmental conditions were recorded and could be viewed throughout the transport **(Fig 3)**. The whereabouts of the samples shipped in the EPS shipper were only accessible via the on-line UPS tracking history (hub locations). There was no record of temperature, location, path, mode of transport, chain of custody, exposure to rough or negligent handling practices, or any indication when or where the package may have been opened or altered.

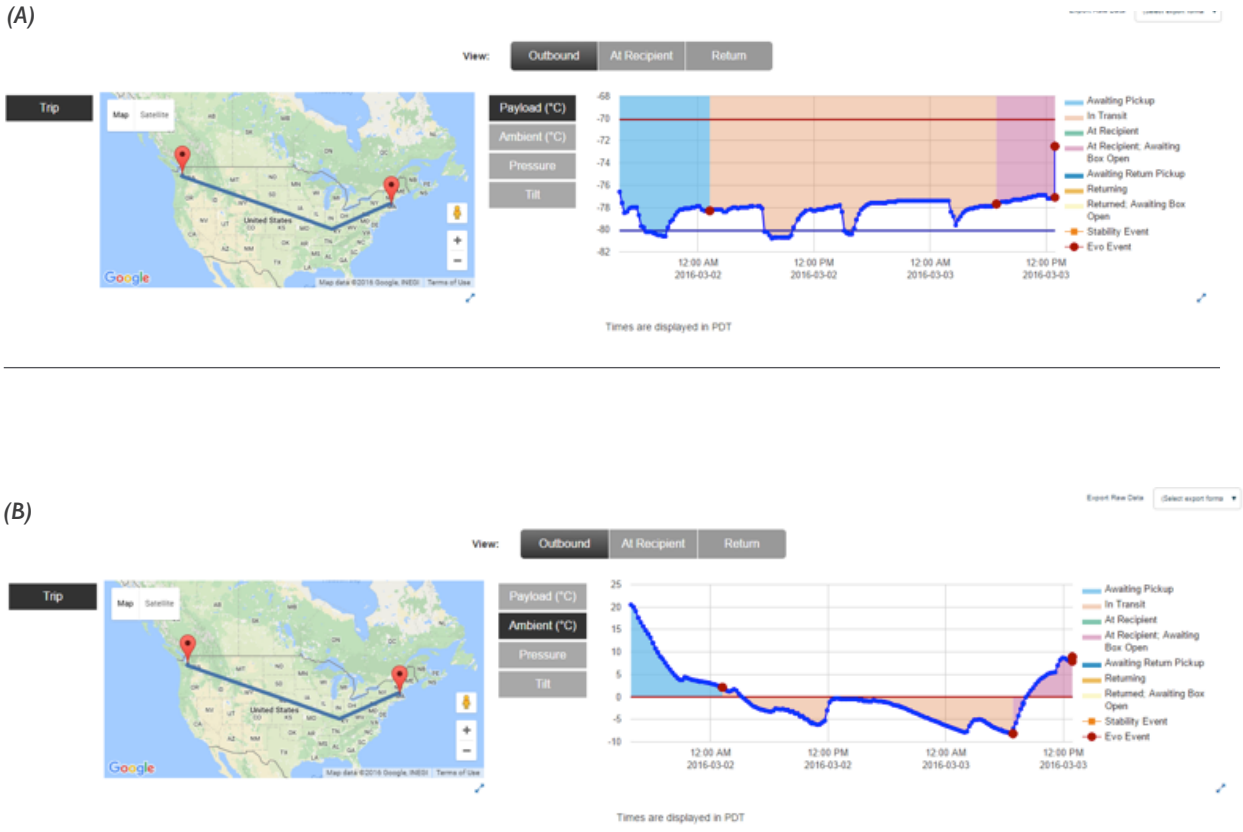

Figure 3. Excerpts extracted from biologistex™ cloud-based SaaS application of the shipment from BioLife Solutions to Brooks Life Sciences using the CRYO evo™ SMART Shipper. (A) Routing and internal payload temperature data. (B) Routing and ambient temperature data.

Discussion

Optimized Cold Chain is achieved by biopreservation Best Practices combined with SMART shipping and logistics. Cryopreservation medium is an essential component of an optimized cold chain management for biological-based therapies and is designed to mitigate the cellular stresses associated with freezing. Maintaining the required cold temperature and product visibility during shipment are also necessary to prevent thermal excursions detrimental to the quality of the therapeutic dose. Insufficiencies in either cryopreservation or shipping can result in significant loss of viability and functionality of the therapeutic cell dose. Historically, these two links in the biopreservation continuum have been disconnected, managed by independent functional groups within an organization, and vulnerable to weaknesses in the cold chain. Recognition of the linkage in the biopreservation cold chain throughout the entire product life cycle, from source material (apheresis/leukapheresis, tissue, blood/marrow) through in-process intermediates to final clinical application, allows for overall risk assessment and risk mitigation to support manufacturing system optimization.

Cryopreservation Medium

Traditional 'Home-brew' media composed of various mixtures of cryoprotective agents (such as DMSO), sugars, salts, and animal/human proteins (including fetal bovine serum and human albumin) have historically been employed for the cryopreservation of primary human cells and research cell lines. However, the animal/human-derived components in such media introduce the potential for disease transmission to patients and may not be amenable to the Good Manufacturing Practices (GMP) guidelines for biologic-based therapies[1]. Furthermore, certain attributes of animal/human proteins including aggregate formation require extra steps of processing to remove particulates[3]. The family of CryoStor® media is formulated to closely resemble the intracellular environment, and is designed to provide enhanced cellular protection during cryopreservation. CryoStor is chemically-defined, devoid of animal/human proteins, and contains a mix of cell permeable and impermeable cryoprotective agents (including DMSO) that provides improved cellular performance post-cryopreservation. CryoStor is also supported by a Quality/Regulatory framework that facilitates inclusion into the manufacturing of cellular therapy/regenerative medicine products. The data presented in **Figs. 2A** and **2B** showcase the enhanced post-thaw viability of Jurkat cells cryopreserved in CryoStor. This study was conducted using a minimum number of samples and shipments and further studies are needed to increase experimental power. However, the significant difference between the results of the two groups is statistically meaningful. Despite statistically similar cell recoveries in all conditions, the difference in post-thaw viability points to the advantages of using an intracellular-like, serum-free media over saline-like, serum-containing media. This also highlights caution to post-thaw analytical methods relying solely on cell number recovery. For non-shipped controls, comparison of CS5 and serum containing groups suggests ~10% improvement in cellular viability at equivalent DMSO concentration. Further post-shipping analysis revealed the viability of the serum-containing group dropped an additional 20% while the CS5 group remained unchanged. A number of factors may have contributed to the loss of viability and function in the cells cryopreserved using "current practices". The impact of using a saline-like formulation for biopreservation has been previously discussed [4]. The concentration of extracellular ions in a saline-like formulated solution during the freezing process can become increasingly toxic to cells, penetrating the membrane and altering the intracellular ionic balance, resulting in signaling disturbances, protein denaturation, and osmotic swelling and lysis upon thaw [4]. The intracellular-like ion formulation of CryoStor is designed to mitigate such stresses during freezing. Other factors contributing to increased stresses and decreased viability during shipment for the CP group may initiate from the EPS box/dry ice shipment inability to maintain a uniform temperature throughout the box, sample position, and temperature profile.

Temperature Monitoring and Clarity of Tracking

Shipping containers incorporating insulated materials such as expanded polystyrene (EPS), expanded polyurethane (PUR) or vacuum insulated panels (VIP) are currently employed for the transportation of some cell therapy products at dry ice temperatures. These methods offer some costs/logistics benefits, as well as introduce some risks related to maintenance of cell product stability. Under conventional dry ice shipping configurations, CO_2 venting often results in the buildup of frost and water on the outer surface of corrugate boxes, which can contribute to complications and delays in delivery, makes handling difficult, and can compromise the physical integrity of a shipment[5]. In addition, dry ice shipments in such boxes can be susceptible to temperature variation as portions of the payload may become exposed to the internal air due to dry ice sublimation during shipment[6]. This is often exacerbated by frequent and improper package orientation during transport and inadequate containment of the product payload within the container. Such conditions can repeatedly expose temperature-sensitive products to detrimental fluctuating temperatures[7] CRYO evo™ SMART Shipper's unique design, materials and product containment ensure the payload is completely surrounded by dry ice at all times, thus eliminating temperature stratification of the product payload. To evaluate thermal performance, we exposed the CRYO evo™ SMART Shipper to cyclic temperature variations that deviated from room temperature (+20 °C) to a high of +40°C and a low of 0°C to mimic potential worst-case scenarios during shipment. The data presented in **Fig. 4**

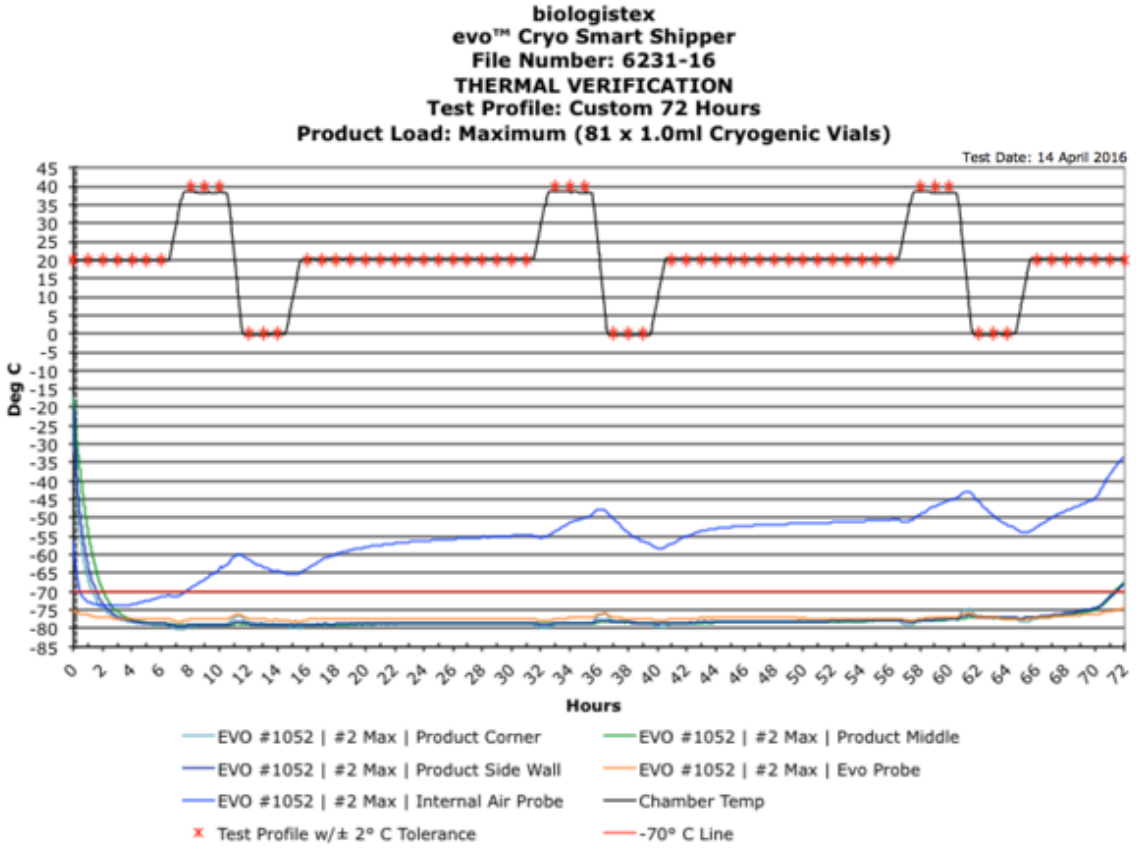

Figure 4. Thermal performance of CRYO evo™ SMART Shipper (typical) showing internal air temperature as dry ice sublimates.

highlights the fact that inherent dry ice sublimation and the resultant increase in the head space inside the box can raise internal air temperature. A condition common in dry ice shipping configurations is when the product payload does not remain completely surrounded by dry ice on all sides, becomes exposed to the internal air and increases in temperature. The results in **Fig. 4** show that the internal air temperature above the sublimating dry ice was averaged -54.3°C and rose to a high of -34.6°C at the 72 hour mark. However, the sophisticated design and high thermal conductivity components of the CRYO evo ™ SMART Shipper completely eliminate the impact of such stratification on payload temperature. **Fig. 4** further demonstrates that, regardless of package orientation or quantity of remaining dry ice, the continuous contact between the dry ice and product payload retainer ensured uniform distribution of temperature throughout the payload to within 1°C (±1°C). As a result, the monitored temperature of the test vials remained below -70°C for 70+ hours with vial-to-vial variability of less than +1.5°C.

Storage & Monitoring

Best practice demonstrates that cell therapies be stored long-term in LN_2 vapor phase freezers (-190°C). It is very important to control, monitor and audit cell therapy storage as it is often the longest duration step of manufacturing, ranging from days to years. Excessive warming during storage can cause irreversible damage to the material which will affect post-thaw quality. The viability/functionality of cells is maintained, and thus maximized, when stored at temperatures below the glass transition temperature of water (T_g, approximately -135°C)[9]. Equally important is that during routine storage/retrievals that innocent samples (the ones not being accessed) are not exposed to warming events that may warm them above -135°C at any point during their storage lifetime (protection of innocence). To ensure preservation and function, cells should be stored in high efficiency LN_2 vapor freezers at -190°C or colder. Access to the freezer should be controlled and monitored. Sample retrieval and resultant innocent exposures should be tracked and recorded. Furthermore, should an innocent sample be unintentionally warmed above T_g, an indication of the event with regards to who, when and for how long, the event occurred should be recorded and the sample owner/researcher notified. Current regulatory requirements dictate that all storage freezers containing therapeutic material must have an autonomous temperature monitoring device with email/cloud reporting installed to verify the freezer temperature is correct and to facilitate instant alarms of any warming.

Traditional LN_2 freezers which have not been specifically designed to facilitate vapor storage modality (such as full diameter lid models) can typically only maintain a top-box temperature as cold as -150°C. This allows very little temperature safety margin during routine innocent exposures (when adjacent samples or even whole racks are accessed). Excessive warming can even occur in high-efficiency -190°C LN_2 freezers when users keep racks outside the freezer for too long. In both these cases the time samples have been exposed to the ambient environment is unmonitored and not recorded during a manual access procedure. A researcher will only know if there has been cell damage after the cells are thawed and tested.

The BioStore ™ III Cryo -190 storage system (B3Cryo) restricts access, monitors and records temperature for all interactions and all samples. This allows cell therapy samples to be controlled and monitored during storage to ensure no excessive warming or unauthorized access has occurred. The B3Cryo also automates the storage and retrieval to greatly improve the ergonomics and safety of working with LN_2 storage.

Conclusions:

The combination of CryoStor® CS5 and the CRYO evo™ SMART Shipper demonstrated superior protection from cryopreservation and transportation stresses, with no measurable decline in structural and functional viability following freezing, 45 day storage and twice 2-day cross-country transits. Moreover, reference (non-shipped) Jurkat T cells frozen in CryoStor® CS5 were similar to, but exhibited less baseline variability, than reference 'home-brew' cryomedia. In contrast, cells frozen in traditional 95/5% cryomedia and shipped in an EPS container experienced a significant decline in viability immediately post-thaw and a delayed return to function post-thaw. The CRYO evo™ SMART Shipper allows real-time status, tracking, and event alarms throughout the entire shipping process, permitting enhanced tracking and knowledge of any environmental excursions as they happen, with the ability to intervene and take corrective or evasive action. The BioStore ™ III Cryo stores cells below -190°C with user access restrictions and monitors all stores/retrievals and temperatures to ensure no samples warm above Tg during their entire storage lifetime. Although this study did not utilize clinical cell products or within a clinical application, and further studies would be required to validate the

risk/benefit ratio of utilizing dry ice temperature shipment for clinical products in comparison to LN_2 dry shippers, this study offers feasibility support to the potential of multiple temperature options for shipment of cryopreserved cell products. Understanding the linkage in the biopreservation cold chain throughout the entire product life cycle - from source material of apheresis/leukapheresis/tissue/blood/marrow, through in-process intermediates, and to final clinical application - allows for risk assessment and risk mitigation to optimize the manufacturing system.

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