Review: The Industry's Need for Closed-System Cryogenic Storage

Erik J. Woods, Ph.D., HCLD (ABB) & Sreedhar Thirumala, Ph.D.

Summary:

The authors present a review of publications regarding cryogenic storage, including historical problems, attempted solutions, and a unique patented resolution. This review highlights the industry's need for a cryogenic storage system that prevents contamination by identifying tragic examples of virus transmission despite technology developments. The authors propose the CellSeal® Closed-System Cryogenic Vial as an advancement in cryogenic storage and position it to meet a critical need in the cell and gene therapy manufacturing process.

Introduction:

Cryopreservation has been developed to store viable biological systems at ultra-low temperatures (≤ –150°C) in a cryogenic medium, such as liquid nitrogen (LN_2), for extended periods of time. At such ultra-low temperatures, all cellular metabolic activities are arrested, such that the systems can be revived and restored to the same living state as before they were stored.

Problem:

Like water, LN_2 can act as a vehicle for the transmission of viruses, bacteria, fungi, and even animal cells. The realization that LN_2 exposed to viruses should be treated as a biohazard is not new (Schafer *et al.*, 1976) and is indicative of the potential contamination problems that LN_2 may present. The submersion of screw cap plastic vials allows for contact between contaminated LN_2 and the sample. At low temperature, condensation of the atmosphere within the vial creates a vacuum that can draw in LN_2. Any contaminants in the LN_2 may contaminate the sample.

A tragic example of this phenomenon occurred when bone marrow stems cells harvested from patients undergoing cytotoxic treatment became contaminated with Hepatitis B virus (HBV) during storage in LN_2 and caused an HBV outbreak by transmission to other stored units (Tedder *et al.*, 1995). Of the six patients affected, human DNA, Hepatitis B surface antigen A, and HBV DNA matching those patients were found in the LN_2. The interesting observation is that DNA from the patients, and thus presumably their cells, was found in the LN_2 indicating that contaminants move both in and out of the storage containers. A follow-up study to this HBV outbreak confirmed the human and HBV sources by DNA sequence analysis (Hawkins *et al.*, 1996).

Other viruses have previously been found to survive direct exposure to LN_2 as well, including vesicular stomatitits virus (Schafer *et al.*, 1976), herpes simplex virus, adenovirus (Jones and Darville, 1989), and papilloma virus (Goodman, 1960; Charles and Sire, 1971). There is also evidence of contamination of LN_2 by other microorganisms, including a wide range of bacterial and fungal species. Fountain *et al.* (1997) conducted a survey of fungal and bacterial contamination of LN_2 freezers used to store hematopoietic stem cells. Of the 583 cultures tested, 1.2% were found to be contaminated by microorganisms. However, four of five freezers examined contained low level microbial contamination, while the fifth freezer was heavily contaminated with *Aspergillus*. The microbial contamination found in the freezers was similar to the microbes found in the contaminated cultures. Though not citing the LN_2 as the microbial source, other reports demonstrate the common occurrence of microbial contamination of cryopreserved stem cells (Prince *et al.*, 1995; Lazarus *et al.*, 1991; Stroncek *et al.*, 1991; Webb *et al.*, 1996).

Previous Solutions:

Until recently, bag storage, despite the potential for failure due to breakage, remained the most secure method to maintain cryostored cells in a closed system. However, bag storage is not amenable for small volumes, such as master cell banks or sperm banks. While screw cap vials have been used for decades for smaller volumes, tests have routinely demonstrated clear risks using these systems. An independent Andrology Department study indicated 45% of cryovials without an O-ring gasket (Nunc Product #340711; Nunc, Nalge International) and 85% of vials with an O-ring (Iwaki Cryovials; Iwaki, Japan) absorbed LN_2 during a three-hour immersion in LN_2 (Clarke, 1999).

One potential way to mitigate LN_2 ingress is to store in vapor-phase nitrogen. However, breakage may still occur as typical containers become brittle even in vapor phase temperatures and virus can potentially remain airborne and still coat adjacent containers (Grout and Morris, 2009). Additionally, temperature fluctuations can be difficult to manage using vapor storage, and temperature gradients in vapor storage tanks can exacerbate this problem. Because the temperature of LN_2 is constant at -196°C, this remains the most robust medium for long-duration cryostorage.

Patented Solution:

CellSeal vials represent a solution to both breakage and container closure in a vial configuration. These vials seal easily using blood-bag-type tubing sealers, and unlike threaded vials, create a true seal. CellSeal vials have successfully passed container integrity testing in accordance with ISO standards to maintain a sterile barrier following storage under LN_2 and following shipment (Woods and Thirumala, 2011). In a recent study, in spite of strict laboratory technique when filling and sealing, we found that 92% of the Corning® cryogenic vials (Corning® USA, Product #430488) and 18% of the Nalgene® cryogenic vials (Nalgene International, Product #340711) allowed leakage of LN_2 during storage. Upon warming, this leaked LN_2 undergoes a 700-fold expansion in volume as it turns into gas, creating a serious vial explosion hazard. In contrast, there was no evidence of LN_2 condensation inside the CellSeal vials when stored in LN_2.

CellSeal Vial Construction:

CellSeal vials are constructed using USP class VI materials chosen specifically for their resistance to chemicals, drainability for maximum cell recovery, and durability under true cryogenic temperatures. The CellSeal vial body is constructed from a commercially available cyclic olefin co-polymer (TOPAS® COC; TOPAS Advanced Polymers, GmbH). COC resins are in packaging materials that deliver pharmaceutical drugs used worldwide (Qadry *et al.*, 2003; Esfandiary *et al.*, 2008; Waxman and Vilivalam, 2009). With a variable glass transition temperature from 70°C up to 177°C (Shin *et al.*, 2005), COC is more break resistant at cryopreservation storage temperatures which helps reduce sample loss during filling, freezing, storage, shipping and retrieval. Being non-polar, COC has low moisture absorption and excellent drainability which prevents aqueous cell suspensions from adhering to the vial surface. It is compatible with a wide range of pH (2-12) and solvents such as alcohols, ketones, and cellosolves. The material is highly transparent and has thermal characteristics that withstand LN_2 exposures.

The CellSeal vial is designed with three independent ports: fill, vent, and retrieval port. The fill tube and vent tube are made of ethylene vinyl acetate (EVA), similar to existing cryogenic bag systems. The fill tube has a fill port that is either a needle or needle-free septum used for filling the vial. This port is sealed using either radiofrequency (RF) or heat sealing after introduction of the sample into the vial. The vent port is an air vent and is fitted with a filter plug made from polytetrafluoroethylene (PTFE) (Porex Technologies, Fairburn, GA, USA) and tested to ensure biocompatibility when used in medical applications (Risbud *et al.*, 2001). The plug provides an ambient pressure fill and acts as a microbial-barrier vent to allow air to escape for easy fluid transfer without allowing the introduction of contaminants while filling or extracting. This tubing is sealed using the RF sealer or heat sealer pre-freeze, then cut open post-thaw above the microbial-barrier vent to aid in fluid extraction. The conical retrieval port has a foil-covered fixed needle septum that is made of EVA at the bottom end of the vial body for fluid extraction post-thaw. The CellSeal vials are the same diameter as conventional cryovials which allows the use of conventional "egg crate" cardboard or plastic separator boxes commonly used for the storage of standard cryopreservation tubes, which can be stored in standard clinical freezers (both vapor and LN_2). Currently, 2 mL and 5 mL CellSeal vials are available in multiple configurations.

Published studies have now demonstrated the closure integrity and sterility maintenance during and after shipping and thawing for cell banking (Woods and Thirumala, 2011). Several groups have used or are now using CellSeal vials for clinical and research cell banking as well as cell and gene manufacturing.

Conclusion

The CellSeal vial is a solution to leakage, breakage, and contamination in cryogenic storage because it is an aseptic closed-system that provides a true seal. It is constructed using USP class VI materials chosen specifically for their resistance to chemicals, durability under true cryogenic temperatures, and drainability for maximum cell recovery. The CellSeal vials' closure integrity and sterility maintenance overcomes screw cap vials' susceptibility to condensation attracting contaminated LN_2. It also provides an alternative to storage bags' tendency to become brittle and break. In conclusion, the CellSeal vial reduces risk and adds control from early stage product concepts through commercialization, making it an ideal solution for the cryogenic storage of valuable biologics in the cell and gene therapy manufacturing process.

About The Authors

Erik Woods, Ph.D., Ossium Health, Inc., Indiana University School of Medicine

Dr. Erik Woods studied cryobiology at the Hillenbrand Center for Biomedical Engineering at Purdue University in West Lafayette, IN where he earned his Ph.D. developing methods for cryopreservation of encapsulated human and canine pancreatic islets. He then completed a Post-Doctoral Fellowship at the Herman B Wells Center for Pediatric Research at the Indiana University School of Medicine in Indianapolis, IN, developing enhanced methods of umbilical cord blood stem cell cryopreservation. Dr. Woods has devoted his career to advancing cell culture and cryopreservation to facilitate mainstream clinical use of cellular therapies. He currently serves as Chief Science Officer of Ossium Health, a company developing the first cryopreserved bone marrow bank from deceased organ and tissue donors, which he also co-founded, and he is a Visiting Professor at the Indiana University School of Medicine.

Sreedhar Thirumala, Ph.D.

Dr. Sreedhar Thirumala received his Ph.D. in Mechanical Engineering from Louisiana State University, where his research was focused on heat and mass transfer in biological systems using mathematical optimization strategies, numerical simulations, and biophysical tests. While formally trained in Mechanical Engineering, Dr. Thirumala has a broad background in interdisciplinary elements of biology including biomaterials, tissue engineering, microfluidics, biopreservation, and bioprocessing technologies. Dr. Thirumala was formerly the Manager and Senior Scientist at Cook Regentec, where he devoted his efforts to translational research and product/technology development with a focus in cell therapy and protein therapeutics.

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Phone: +1 317.917.3450
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