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Cell Snapshot

Uniformity in cryopreservation is a necessary step towards suspending cell function in time post-thaw.

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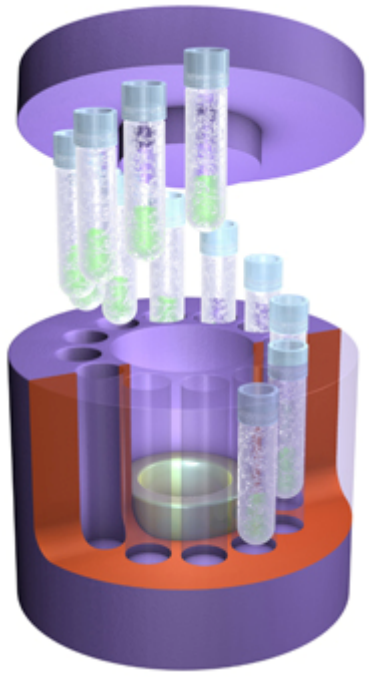
Introduction

By virtue of the complexity of the living organism, the life sciences are faced with subjects of study that present a seemingly infinite number of variables, and yet effective experimentation requires significant simplification. Molecular biological studies of multi-cellular organisms frequently resolve to the study of cultures of individual cells isolated from tissues, with the presumption that the molecular behavior of the individual cells duplicates the same response that would be expected if the cells were in the multi-dimensional environment of an organism.



Insulation-based SSC Design of CoolCell.

Although cultured cells may certainly provide an approximation of biological function, they are subject to the selective pressures of the environment. In time the culture will become populated by those cells best capable of surviving the stresses of laboratory life, leading to a drift in cultured cell performance. As a result, meaningful and reproducible experimental results become linked to the suppression or, at the very least, uniform control of the influences that contribute to cell culture drift.

Freezing Time

Cryopreservation is a critical component of cell culture work. The cells which survive the thermodynamic journey from the warm temperature of the incubator to the -196 C

environment of the liquid nitrogen storage tank are free from the influences of the clock. This capability provides the cell culturist with a means of taking a snapshot of the culture at a given time in its history.

Until the sample aliquots from a given freezing session are exhausted, the culturist has the option of going back in time and starting the culture again as it was when the freezing step was performed, and by this technology has a means to counter the effects of cell culture drift and cell division number limitations. The ironic cost of this capability derives from the fact that the journey to and from the cryogenic state presents a series of stresses that can impose selective pressures far more extreme and influential than those to which the cells would be exposed to in culture. As a result, the surviving cells emerging from a non-ideal cryogenic preservation sequence can be the exclusive members of a subset that do not effectively duplicate the original culture.

The challenge of cryogenic storage is, in a word, ice. Cells are approximately 70% water, and when chilled to below the freezing point, ice crystals will form in the cell interior, lethally disrupting the intracellular structures. Cryogenic storage methods are successful only because the process includes a reduction of the intracellular water content prior to freezing, and, with the added benefit of a cryoprotectant, is successful in sufficiently limiting ice crystal growth. As the freezing process initiates in the extracellular fluid space, the forming ice crystals exclude and concentrate the dissolved solutes.

As the freezing progresses, the cells are confined to a decreasing volume of solute solution that is increasing in concentration. The increase in extra-cellular salt solution concentration promotes an outflow of water, effectively dehydrating the cell. When the extracellular solute solution approaches the eutectic phase transition temperature, the remaining liquid will solidify, as will the now dehydrated cells.

The degree of dehydration of the cell is a key parameter that is controlled by the rate of temperature decrease. If the rate of temperature reduction is too low, the cells will become dehydrated beyond the critical water content survival limit due to prolonged exposure to the exterior concentrated salt solution. In addition, the added time spent in the high salt concentration environment can have a deleterious effect on cell health through exposure to inappropriate pH, toxic ion levels and concentrated solute-induced cell surface protein denaturation. Conversely, should the rate of temperature reduction be too great, the cell interior will supercool and ice crystals will nucleate, initiating interior ice crystal growth while the interior water percentage is still dangerously high.

The two opposing boundary conditions restrict the freezing rate associated with a peak of cell viability to a narrow range. The value for the optimal freezing rate may vary with cell type and is dependent upon both cell size and membrane permeability Fortunately, for a large portion of cultured mammalian cell types, in the presence of common cryoprotectants such as DMSO, the optimal freezing rate will coincide with a value of -1 C/min, and any means of reliably attaining this rate of freezing will be beneficial in the cryopreservation process.

Control Crossways

There are two main avenues for achieving a controlled rate of cell freezing. The first and most flexible of which involves the use of microprocessor-controlled refrigeration systems that can be programmed to follow a pre-determined profile of temperature reduction. Although these units offer easy modification of the desired freezing profile along with greater options with regard to capacity, the primary drawbacks are the size and the cost of the instruments, which is on the order of several thousands of dollars.

In addition, the reality of practical laboratory research more often includes the frequent requirement for cell freezing capability by multiple researchers, and ideally the minimum number of freezing stations loosely parallels the number of tissue culture hoods occupied on an average workday.

The second avenue leads to the use of passive freezing units, which exploit the consistent thermodynamic principles of temperature differentials and thermal conductivity. Starting with a reliable thermal sink such as a -80 C deep freezer or dry ice locker (-78 C), cell vials can be encased in a device that will, through an appropriate combination of thermal capacity and insulation, provide a freezing profile with the desired temperature reduction rate. Until an alternative became available, the method for insulating cryovials during the freezing process defaulted to the inventiveness of the individual researcher.

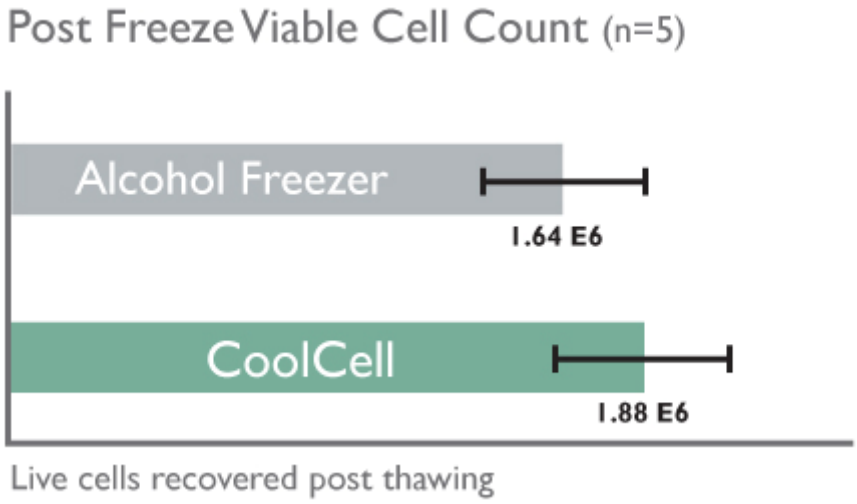


Figure 1. High post-thaw cell viability with CoolCell. HUVEC cells were resuspended in freezing medium at a concentration of 2x10^6 cells per ml. 1 ml aliquots were portioned into 1.8 ml Corning cryovials and frozen at -1C per minute in either a BioCision CoolCell or in an alcohol-filled cell freezing unit. Five vials frozen by either method were rapidly thawed and resuspended in growth media. Live cell count were obtained by the trypan blue exclusion method.

Numerous protocols for cell freezing include steps such as wrapping the vials in paper towels, cotton or tissue, or encasing the vials in recycled styrene foam tube racks. A common acceptable threshold for the success of these freezing methods is that sufficient cells be recovered alive upon thawing to repopulate a culture flask within a reasonable timeframe, while dismissing the fact that such methods can result in cell cultures populated by a subset of the original culture. The selective influences imposed

upon the frozen cell sample can result in a wide variation in cell functionality and, in the worst case, lead to unrepresentative cell performance, assay results, biomarker behavior or cell-based diagnostic parameters.

Alcohol-Filled Systems

An improvement in the thermal flow control for the passive freezing profile method became available in the form of alcohol filled containers into which the cell vials are placed, separated from direct contact with the alcohol by means of a plastic barrier. The alcohol-filled systems rely on a large thermal mass and high heat transfer to slow the sample cooling rate to approximately -1 C/min. These insulation-free designs depend upon the thermal conduction limits of the alcohol and the heat transfer limits of the air inside the freezer to regulate the heat flow, in effect controlling temperature reduction by temporarily overwhelming the heat removal capacity of the freezing unit.

The heat lost from the alcohol (250 mL) is approximately 10x greater than the heat removal required for sample freezing, placing a greatly amplified thermal burden on the refrigeration system that has the potential to cause temperature fluctuations in locally stored archived samples. The negative impact upon archived samples due to repeated temperature cycling is avoided in diligent laboratory practice by assigning a common and remote region of the freezer to the cell freezing process. This practice, however, imposes a secondary concern in that busy laboratories can often require the freezing of samples generated by multiple researchers, and the combined heat from two or more alcohol freezing containers in the same location will significantly alter the temperature reduction profile of all containers present.

Alcohol-filled freezing containers also require that the alcohol be changed every five uses as absorbed moisture and evaporation can alter the heat capacity of the system and thereby cause variance in the thermal profile. In addition to the cost, the alcohol replenishment results in continuous generation of contaminated solvent that must be removed through hazardous waste streams. In daily practice, tracking the number of use cycles requires vigilance and, as most alcohol freezing units are laboratory community property, the consistency in maintenance descends to the performance level of the least diligent lab member.

Likewise, mistaken replacement of the alcohol with an alcohol other than the required isopropanol is a repeated error made by researchers unmindful of the fact that different alcohols have significantly different heat capacities and that switching alcohols will alter the freezing profile.

A recent alternative to alcohol-filled freezing containers is found in the radially-symmetric insulation solid-state core (SSC) based design of the BioCision CoolCell product, which takes advantage of the combination of precision insulation geometry and small solid core thermal ballast (Figure 1).

The core has a total heat capacity that is approximately 7% of the alcohol-filled container system. The total heat capacity of a fully loaded unit is less than that of a typical freezer box of samples, therefore the freezing unit can be confidently placed next to previously archived samples without imposing a damaging thermal fluctuation. As the physical positioning of the insulation and the heat capacities of the insulation and solid core materials are unalterable, when placed into the constant temperature environment of a typical regulated deep freezer, the contained samples will experience very consistent freezing profiles (Figure 2).

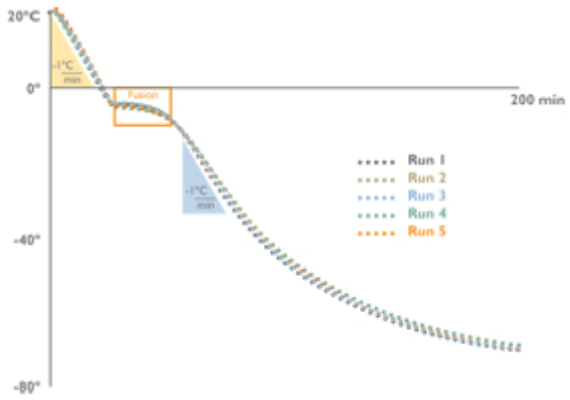


Figure 2. Highly reproducible freezing profiles with CoolCell. A 1 ml volume of cell freezing media was placed into12 Cryovials. A thermocouple probe was introduced into one vial in an axial orientation with the probe end at the center of the liquid volume. All vials were equilibrated to 20 C, then loaded into a BioCision CoolCell and placed into a -80 C freezer and internal vial temperature was recorded by a data logger at 10 second intervals. After a 4 hour freezing cycle, the vials were removed, thawed and again equilibrated to 20C. The repeatability of the temperature profiles is shown with five consecutive freezing cycles. Click to enlarge

Moreover, the unit can be used repeatedly and indefinitely with no maintenance beyond insuring that it is dry at the time of sample loading. This simple and widely used method allows researcher to perform cell cryopreservation with repeatability and uniformity in freezing rate and post-thaw performance profiles (Figure 3).

It is worthy to address the final stage in the freezing process for all freezing units: the transfer step to the liquid nitrogen archive. Upon the opening of a freezing device, the vials contained therein are exposed to the room temperature atmosphere. As the rate of heat transfer for any system is dependent upon the temperature differential between the immediate and final temperature of the transition, the approximately 100 C temperature difference will impose a rapid temperature shift in the vial contents.

Thermometric tests under these conditions using typical cryovials containing 1 mL of freezing media have demonstrated a rise in temperature to -50 C in <1 min. Temperature cycling can be particularly damaging to frozen cells as smaller and inconsequential intracellular ice crystals have an opportunity to reform, combine and extend under these relatively warmer temperatures. For this reason, it is recommended that upon the removal of vials from a freezing device, that they be immediately placed on dry ice until the remaining steps of transfer to long-term archival storage can be complete.

Conclusion

In summary, cryopreservation of cultured cells is a proven and essential process that provides the researcher with a means of protecting and sharing essential investigative materials. The preservation of samples of PBMCs, stem cells, patient cells, cell lines and other investigative cell material is of compromised value if a hard to standardize method of cryopreservation imposes variable and unpredictable influences on the constituents of the emerging cell population. Precision-engineered insulation cell freezing containers such as CoolCell represent standardizable means of providing reproducible cell freezing profiles (Figure 2 and 3). Properly applied, these simple devices can contribute greatly in assuring that valuable experimental assets are not only preserved, but yield to provide consistent and meaningful results.

References:

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