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Chapter 1

Cryopreservation of Adherent Cells on a Fixed Substrate

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1. Introduction

Tissue culture has become the backbone of research in the life sciences. Large numbers of primary cells and established cell lines are available from the American Type Culture Collection (ATCC, Manassas, VA) and various other sources. Some cell types are relatively easy to grow and maintain in culture while other cell lines have specific handling issues that must be learned. These cells come from a variety of animal and human sources and are being used for research as well as the production of enzymes, hormones, and other biological compounds. Toxicology applications range from the use of cells as indicator systems for environmental pollutants to screening cosmetic formulations, new drugs and household chemicals for potential risks. Such biodetector systems may involve cells from almost any vertebrate or invertebrate species. In addition, the universal demand for a reduction of the use of animals in research has prompted the development of alternative methods to determine applicability of new products or procedures. New technologies such as high-throughput screening have been designed to reduce animal testing while increasing the rate at which new drugs or products are brought to market. It has become increasingly clear that cell–based assays will provide the most information for these technologies to be effective in preliminary screening of potential new drugs and products [1].

Convenient cell based systems for a wide variety of applications using a variety of cell types representing key cell-types in various tissues are needed. Readily available cells on a fixed substrate, such as a microtiter plate, that can be used with a minimum of preparation time would not only decrease costs, but also increase the number and variety of assays performed and potential products that are screened in a given amount of time. The idea of cryopreserving cells on a fixed substrate is not new. Researchers have been attempting to cryopreserve cells on a fixed substrate for some time using a variety of cells [2-9]. In general, cell survival in these studies was lower that when the same cells were cryopreserved in suspension. Still, a method has not been developed that can be applied to many different cell types and applications.

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Most cells used in research are cryopreserved after addition of 10% dimethyl sulfoxide (DMSO) to cells in suspension in cryovials, slow rate cooling, with or without induced nucleation, and storage at-80°C or below-135°C. For most applications, this procedure is adequate to provide viable, usable cells whatever the yield upon thawing. Often no consideration has been given to how the cryopreservation process affects the cells and could potentially affect their function upon thawing. Not only physical changes, such as water nucleating to form ice, but also chemical changes take place as the temperature is reduced that subsequently affect the viability and survival of cells and tissues upon thawing. As the temperature is reduced, heat is removed and molecular processes are slowed which leads to a variety of structural and functional changes within the cells even before freezing. As a consequence, the cell experiences a cascade of biochemical and biophysical changes that sensitize the cell to further injury and can lead to irreversible damage. For example, the rate at which cells are cooled and rewarmed is known to be a major determinant of cell survival. As the temperature of the system is reduced, ice forms initially in the extracellular space. Pure water separates as ice crystals so that solutes are concentrated in the remaining liquid phase. As a consequence water moves out of the cell across the plasma membrane in an effort to reestablish osmotic equilibrium within the extracellular space. If the cells are cooled too rapidly, less time is allowed for water to move out of the cells and intracellular ice occurs causing irreparable cell damage. If cells are cooled too slowly, more water is allowed to leave the cells increasing the solute concentration within the cell (Figure 1). This increase in solute concentration both inside and outside the cell has been termed "solution effects" injury because it encompasses a number of changes that include increased salt concentrations, which can denature proteins and membranes, precipitation of buffers, induced pH changes, increased concentration of proteins may result in cross linking, or simply removal of structurally important water. Usually an intermediate cooling rate provides the best compromise between cytotoxicity due to cryoprotectants (CPAs) and ice formation. During rewarming the process is reversed, ice is replaced with water, and cryoprotectants (CPA) are removed from the system. However, physical and chemical changes to bring the cells back to physiologic temperature can still cause damage. As the sample is warmed recrystallization can occur. Recrystallization is when metastable ice crystals that formed during freezing are given an opportunity to reform larger crystals during rewarming. These ice crystals can cause damage to the cells in a similar manner as those crystals that were formed during freezing. Another concern during rewarming is the removal of the cryoprotectants. The CPAs were added to the samples prior to freezing and cell permeating compounds like DMSO, replace cell water further decreasing the risk of ice damage. However, during rewarming, DMSO does not move across the cell membrane as readily as water. An imbalance can develop so that the cells will tend to take up water faster than the DMSO is removed causing swelling. Too much swelling can cause irreversible cell damage if the rewarming is not controlled appropriately, even if the freezing protocol worked. All these factors affect the overall survival of cells during cryopreservation. Some cells can be cryopreserved readily, such as hematopoietic stem cells and fibroblasts, while other cell types are much more difficult, such as hepatocytes. Therefore, optimization for a given cell type is usually required, particularly if they are adherent [1,10-12].

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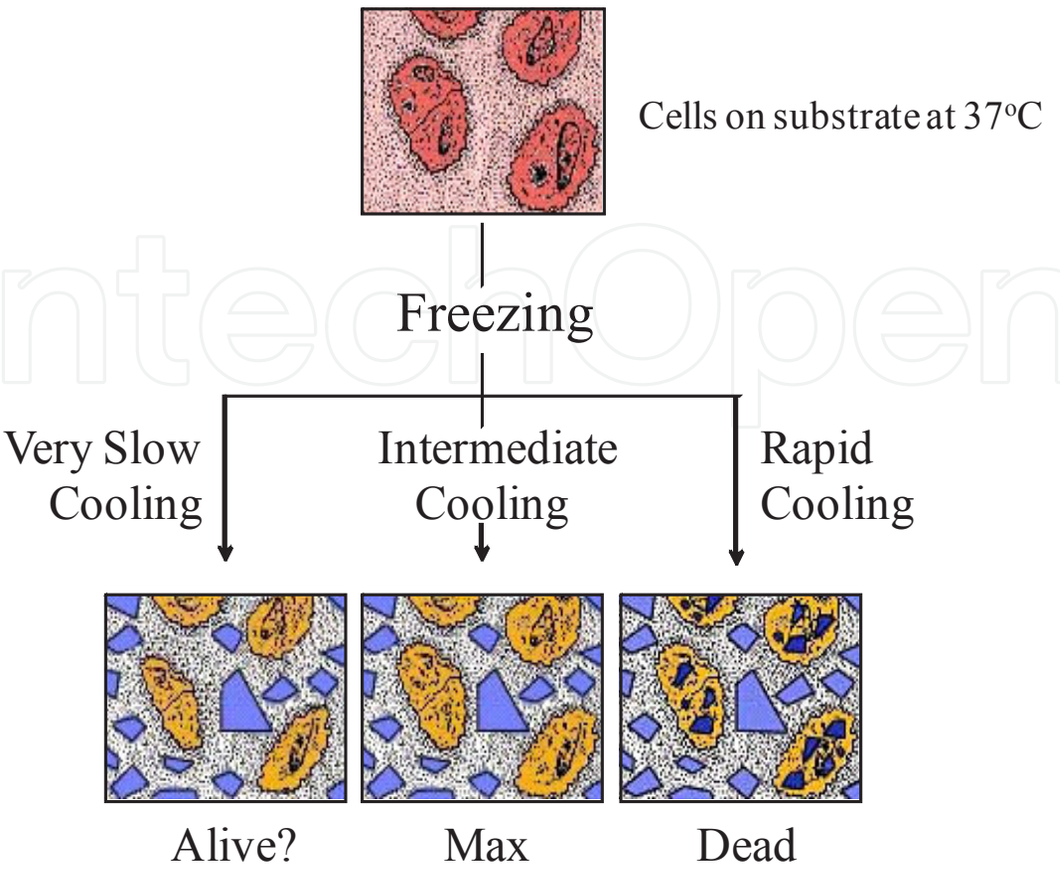


Figure 1. Comparison of differing cryopreservation strategies. If the cells are cryopreserved by freezing ice forms (blue crystals) initially in the extracellular environment and the cells undergo cooling rate dependent shrinkage due to osmotic dehydration. The slower the cooling rate the longer intracellular water has the opportunity to move out of the cell by osmosis due to the increasing osmolality of the extracellular environment as water is incorporated into ice crystals. Maximum cell viability is usually achieved at an intermediate cooling rate that balances osmotic dehydration and the risk of intracellular ice formation. Rapid cooling permits intracellular ice formation and usually leads to cell death upon rewarming. Very slow cooling may lead to excessive cell dehydration and cell death.

In addition to the physical and biochemical changes that take place within the cell during cryopreservation, there is the added parameter of attachment to a substrate that must also be taken into consideration. As described in this chapter, considerable time has been spent developing procedures to cryopreserve cells on a fixed substrate, microtiter plates in particular. However, the protocols described below can be applied to cells fixed on other types of substrates such as a glass slide or a three dimensional scaffold for the purpose of making a tissue engineered construct. The purpose of the CryoPlate™ concept is to provide cells on plates that are ready to use for any number of applications, toxicology testing being one of many. Ideally, any cell type of interest could be cryopreserved on plates and then be ready to use with a minimum of processing time. In practice, no two cell types behave exactly alike when cryopreserved, however, basic criteria and protocols can be established and then adjusted and optimized to fit a specific cell type. While strict criteria have not been established, some basic parameters, specifications, were necessary to guide the development of the CryoPlate™ concept. These are (1) uniform cell number in all the wells within the microtiter

plate (2) >80% of the cells in all the wells are viable (3) the specific activity of a given cell type is intact (4) reproducibility; the retention and viability of the cells is similar in independent experiments after cryopreservation (5) ease of processing; the steps involved in thawing the plate and readying the cells for a specific assay should be user-friendly, not requiring special handling, and not be time-consuming. Potentially any adherent cell type could be established within our system including modified cell lines that may contain plasmid constructs. Such a system would provide cells that are ready to use with a minimum of preparation time and would also be conducive to automation and miniaturization.

2. Materials and methods

Cells: Two cell lines were used in these studies. One comes from rat thoracic aorta called A10 (ATCC# CRL-1476), and the second comes from bovine corneal endothelium, BCE (ATCC# CRL-2048). Both cell types were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS) at 37°C with 5% carbon dioxide.

Cryopreservation Methods: For cryopreservation experiments, different cryoprotectant formulations, the plates were placed on ice and increasing concentrations of cryoprotectant(s) added. Then the final cryoprotectant solution was added and left for 10 minutes on ice before being cooled at a controlled -1.0°C/min. rate to –80°C with or without nucleation at-6°C as described in the text. The plates were stored at ≤–135°C in vapor phase nitrogen or in a mechanical storage freezer. The plates were thawed rapidly at 37°C using a water bath for single step thawing. For the two step thawing, plates were left at an intermediate temperature before rapid thawing at 37°C using a waterbath or some other device as described (US Patent #6,596,531) [17]. The cryoprotectant solution was then removed, the wells washed with 0.5M mannitol and culture medium before being left in media for a 1 hour recovery period at 37°C in a tissue culture incubator.

Measurement of Cell Viability and Proliferation: Cell metabolic activity will be assessed using the resazurin reduction assay (alamarBlue™) [13]. Reduction of resazurin to resorufin measures the oxidation/reduction reactions taking place within cells. Resazurin was added directly to the wells in culture medium and incubated for 3 hours at 37°C. Upon reduction of resazurin to resorufin a color change occurs and this color change can be measured and quantified. The culture plates were read using a Gemini EM fluorescent microplate reader (Molecular Dynamics) at an excitation wavelength of 544nm and an emission wavelength of 590nm. Viability is expressed either in percent of untreated controls or after correction, using relative DNA content, for cell losses. In addition to measuring viability after rewarming, the ability of the cells to proliferate was also examined. Because resazurin is non-toxic, it can be used repeatedly without harming the cells [14-16]. Increased metabolic activity over time is indicative of proliferation and decreased metabolic activity is indicative of cell death by apoptosis, subject to verification as described below.

DNA Measurement: The proportion of cells remaining in the well of the microtiter plate after cryopreservation can be assessed by measuring the DNA content of the cells within a given

well. The Cyquant assay (Molecular Probes) uses a fluorescent dye to label nucleic acids that can then be measured using a fluorescent microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The assay includes a step using RNAse A (Sigma) to eliminate the variable amount of RNA within individual cells and thereby provide a direct measure of the DNA content of the cell alone.

Temperature Measurement: The temperature profiles for individual wells in a microtiter plate were made using thermocouples placed in the middle of wells at the corners of the plate and one well in the center. Several well configurations were evaluated including a 6 well plate where each well contained a thermocouple. The cryoprotectant solution used for these measurements was 1-2M DMSO. Temperature measurements were taken using a datalogger (Omega). Measurements were made every 5 seconds to 5 minutes depending on the rate of warming or cooling being measured.

Evaluation of Apoptosis: Adherent cells were cryopreserved using our protocol and apoptosis was evaluated using Nexin staining. Nexin stain binds to phosphatidylserine that translocates to the outside of the membrane during apoptosis. After thawing, cells were allowed to recover for 1 hour at 37°C. Cells were detached from the bottom of the well and then incubated with nexin stain for 20 minutes at room temperature. Then the samples were run through the Guava cell analysis system which counts the cells and measures the staining in a similar manner as a flow cytometer [16].

Statistical Methods: All experiments were performed several times. The statistical analysis used included t-test, ANOVA and logistic regression methods as appropriate for the type of data being analyzed.

3. Results

3.1. Initial protocol setup

Simple cooling of a commercial microtiter plate by conventional means results in variable thermal profiles. The objective is to have uniform conditions across the plate such that the thermal history of the cell populations in each well of the plate is not significantly different from each other. Conventional wisdom in cryobiology has established that cell survival is markedly influenced by thermal history and the temperature profiles experienced during cooling and warming.

Cryopreservation experiments using adherent A10 cells were initially performed in a fashion similar to that typically done with ampoules of suspended cells and two assumptions were made. First, adherent cells can be cooled successfully at a similar rate as the same cells in suspension,-1.0°C/minute. Second, that thawing should proceed as rapidly as possible at 37°C, the same as for cells in suspension. A10 cells were exposed to 2M DMSO then cooled at -1.0°C/minute to -80°C before being stored overnight at <-135°C. Thawing was performed by placing the plate in a 37°C water bath with the water touching the bottom of the plate. The plate was then placed on ice, DMSO was removed and saved along with subsequent washes to check for

potential detachment of cells and whether detached cells were viable. Both the supernatants and any cells in the wells of the plate after rewarming were checked for viability using the metabolic indicator alamarBlue™.

Measurement of viability after thawing demonstrated essentially no cell survival. When these same cells were cryopreserved as cells in suspension using the same concentration of DMSO their viability was >70%. Several observations were made during this experiment. It was noted that when the plate cooled the plastic constricted and contorted, changing the shape of the plate, which then reversed itself upon rewarming. Most of the cells that had been attached prior to cryopreservation were no longer attached after rewarming and were no longer viable when assessed for metabolic activity.

In an effort to retain cells on the plate and maintain their viability, a two step warming protocol was investigated. This assumed that the differential thermal properties of tissue culture plastic and the adherent cells are influencing the extent of cell recovery. Particular attention was paid to the rates of heat transfer during freezing and thawing. Since, in general, slow cooling is mandatory for optimum survival of cryopreserved mammalian cells, the subsequent experiments focused on the effect of warming conditions following cooling at a standard rate of -1°C/min.

A two-step warming protocol was shown to improve the cell recovery in this adherent cell model [17]. Frozen plates were removed from <-135°C storage and placed at 23°C in air until the cultures reached 0 to -70°C. Sample temperature was recorded in representative wells in the 96 well plates. Plates were then placed into a 37°C water bath to complete the thawing process as rapidly as possible with due care not to allow the wells to warm beyond 0°C. The plates were then immediately placed on ice, washed, and left in culture medium for assessment of metabolic activity. Results from an experiment in which the temperature for transition from step 1 to step 2 was varied are shown in Figure 2. If the plate was rewarmed to -70°C and thawed rapidly at 37°C, there is little difference in viability as compared to a single rapid thawing step. However, allowing the plate to rewarm to progressively higher temperatures resulted in improved viability with the best viability measured when the plate was allowed to warm to -20°C before rapid thawing at 37°C. It was also noted that the more gradual rewarming to -20°C allowed any distortion that occurred due to the plate being cooled to such low temperatures (<-135°C) to subside so that when rapid thawing commenced, no sudden structural changes in the plate occurred.

Using this new two step warming regimen, a preliminary experiment was done to evaluate the effect of DMSO concentration using the same cell model, A10 cells. DMSO (0-5M) was added to wells containing $2.5x10^4$ cells and the plates were cooled and stored as previously described. A representative experiment in which the cells were warmed to –20°C in step 1 of the warming protocol is shown in Figure 3. 1-2M DMSO provided the best protection for adherent A10 cells against freezing-induced loss of viability. These experiments yielded approximately 25-35% of the original cell viability prior to cryopreservation.

While viability improved with 1-2M DMSO, a greater increase was desired. However, this series of experiments did demonstrate that adherent cells could be cryopreserved on plates.

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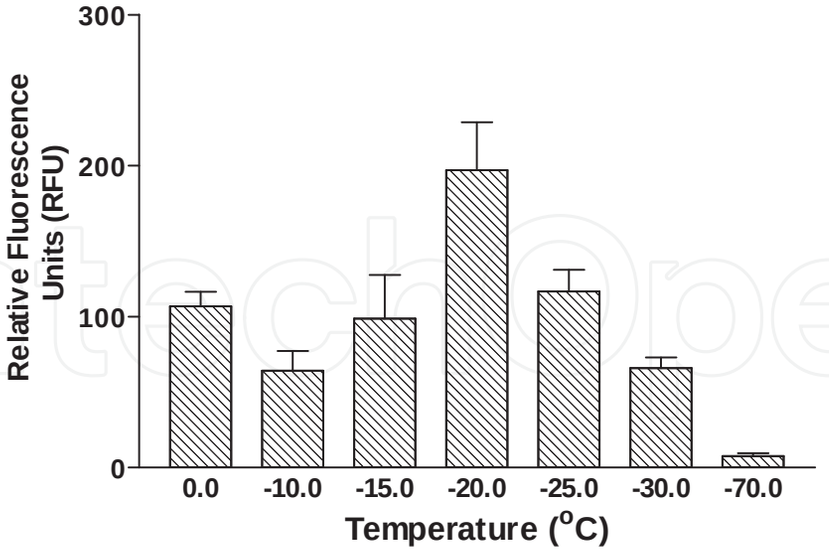


Figure 2. Cell viability after varying the transition temperature from slow to rapid warming of cryopreserved cell cultures. The viability of A10 cells was assessed after cryopreservation in 1M DMSO. Plates were removed from –130°C and placed at ambient temperature (23°C) for slow warming to temperatures ranging from 0 to –70°C, whereupon the plates were transferred to a 37°C water bath for rapid warming to ~ 0-4°C. Then metabolic activity was measured using alamarBlue™.

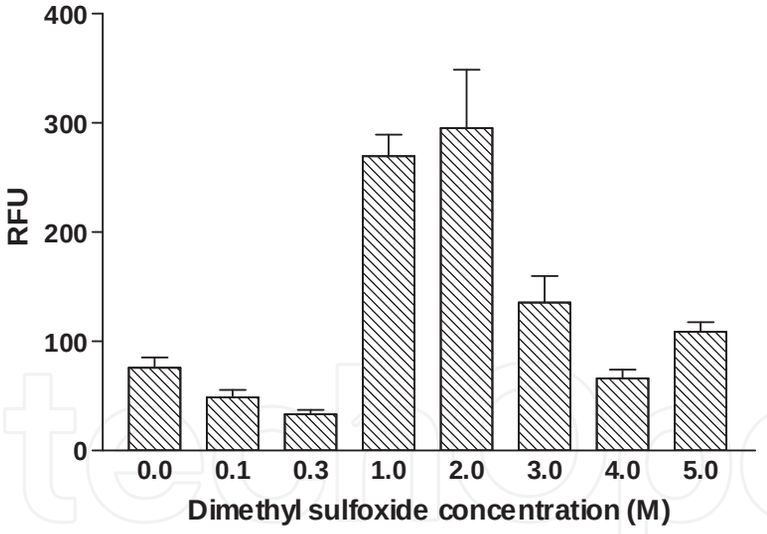


Figure 3. Cell viability of adherent vascular smooth muscle cells cryopreserved in the presence of varying concentrations of DMSO. A10 cells were plated, exposed to varying concentrations of DMSO and cooled at-1°C/min to –80°C then stored at –130°C. The plates were warmed in two steps as described in the text and metabolic activity was measured.

Further work was required to optimize the protocol to improve cell viability after cryopreservation and also, to evaluate and optimize the viability using an entire plate. With the advancements in high throughput screening and the push to reduce the number of animals used in research, the ability to cryopreserve cells across an entire multiwelll plate would further the development of cell-based assays providing suitable alternatives to animal testing.

3.2. Warming rates

Having developed a warming protocol that retains cells on the plate and maintains their viability, attention was then paid to optimizing the warming protocol for a complete multiwell plate. Initial observations of cell viability across a plate (Figure 4) after rewarming demonstrated regional variations in cell viability with the highest survival being recorded from wells at some edges of the plate.

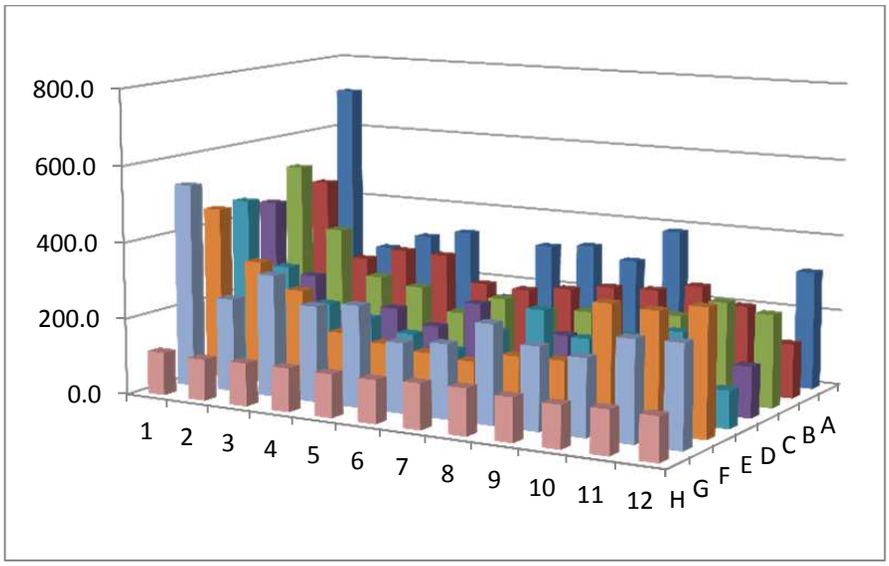


Figure 4. Variability of cell viability in a 96-well plate. Cells were cryopreserved in 1M DMSO. The plate was warmed at 23°C in air to –20°C and then warmed rapidly in a 37°C water bath. The plate was then placed on ice and the cells were washed to remove the DMSO. Cells were left in culture media and alamarBlue™ for assessment of metabolic activity. The results are typical of several experiments. Data from wells with cells minus background in rows A-G. The control wells (background) without cells were in row H.

This effect might be explained by the orientation of the plate during cooling in the control rate freezer leading to differential thermal conditions at various locations in the plate. However, experiments done that evaluated the orientation of the plate within the freezer demonstrated that edge effects were due to the plate and not where the plate was located in the control rate freezer (data not shown). Since warming conditions had already been indicated to be a determinant of cell viability, measurements of the thermal history of different wells in a typical frozen plate during rewarming were measured.

Thermocouples were placed into wells at each corner of the plate and in one well at the center of the plate. Temperature profiles were measured every 15-60 seconds using a datalogger (Omega). The plate was stored at >-130°C overnight. The next day, the plate was rewarmed at ambient temperature (~25°C). The well at the center of the 96-well plate had a different warming profile compared with wells at the outer corners (Figure 5). Uniform conditions of freezing and thawing are necessary in order that optimum conditions for cell survival are applied consistently to each well in the plate.

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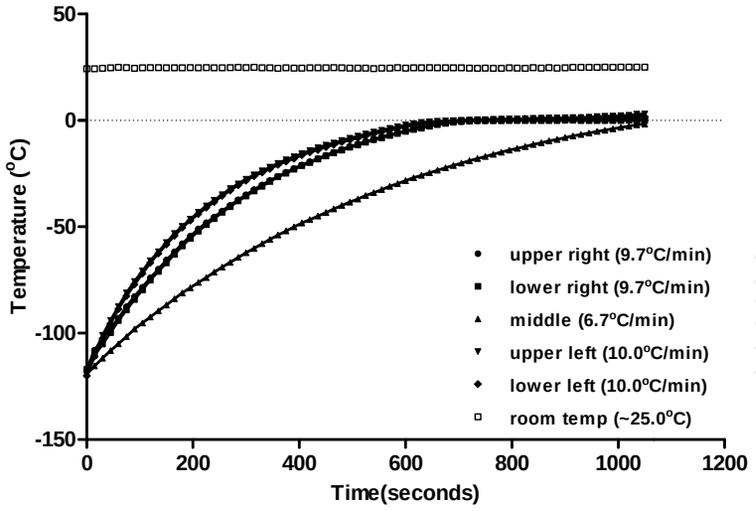


Figure 5. Warming rates at various locations in a 96 well plate. Each well was filled with 150 µl of 1.5% agarose and allowed to solidify. Warming rates were determined using thermocouples placed in the corners and a middle well of a 96-well plate. Warming rates were determined by recording temperatures every 15 seconds using a datalogger (Omega) that records the temperature from all the thermocouples simultaneously.

Warming the plates uniformly proved to be a challenge. An adjustment was made to the initial two-step warming protocol to simplify the procedure. Originally, the two-step protocol called for removing the plate from the cryofreezer and allowing it to warm in air at ambient temperature to –20°C in the first step. This procedure was cumbersome from the point of view of having to carefully monitor the warming process to –20°C in air to ensure there was no thermal overshoot. This problem was solved by placing the CryoPlate™ in a –20°C freezer for the intermediate warming step. An equilibration time of 30 minutes was used and was based on temperature measurements made that defined this time period as the minimum period required to equilibrate the plate to -20°C. The next step was to thaw the plate quickly at 37°C. With this modification, the intermediate temperature of -20°C was reached without the danger of thermal overshoot and it also simplified the warming protocol. It also had the added benefit of establishing a consistent temperature across the plate before the rapid warming step, so that any variations during rapid warming across the plate were kept to a minimum. Steps were also taken to improve thermal conductivity and provide more heat transfer between the CryoPlate™ and the environment by employing custom-made heat sinks and thermal conductive paste. Aluminum heat sinks designed to fit flush with the bottom of the microtiter plate were used for the equilibration at –20°C and for the rapid thawing at 37°C. The wells of the plate were filled with 50 µl of 1M DMSO in PBS and cooled to ~-130°C. The plates were then allowed to equilibrate in a –20°C freezer for up to 30 minutes, and then the plate was placed at 37°C for rapid thawing to the termination temperature of 0°C. Temperature measurements were made and warming profiles were determined as previously described. As mentioned previously, distortion of the plate occurs during cooling. This distortion compromised the tight fit with the platform of the heat sink. To counteract this shape change, we used a thermal conducting compound (Wakefield Engineering) to interface between the plate and the heat sink. The marked effect of this modification is illustrated in Figure 6, where it is seen

that significantly improved uniformity was achieved using the heat sinks in conjunction with the thermal compound. The large disparity in warming rate previously observed between the edges and the middle of the plate was alleviated using the heat sinks with the thermal conduction compound.

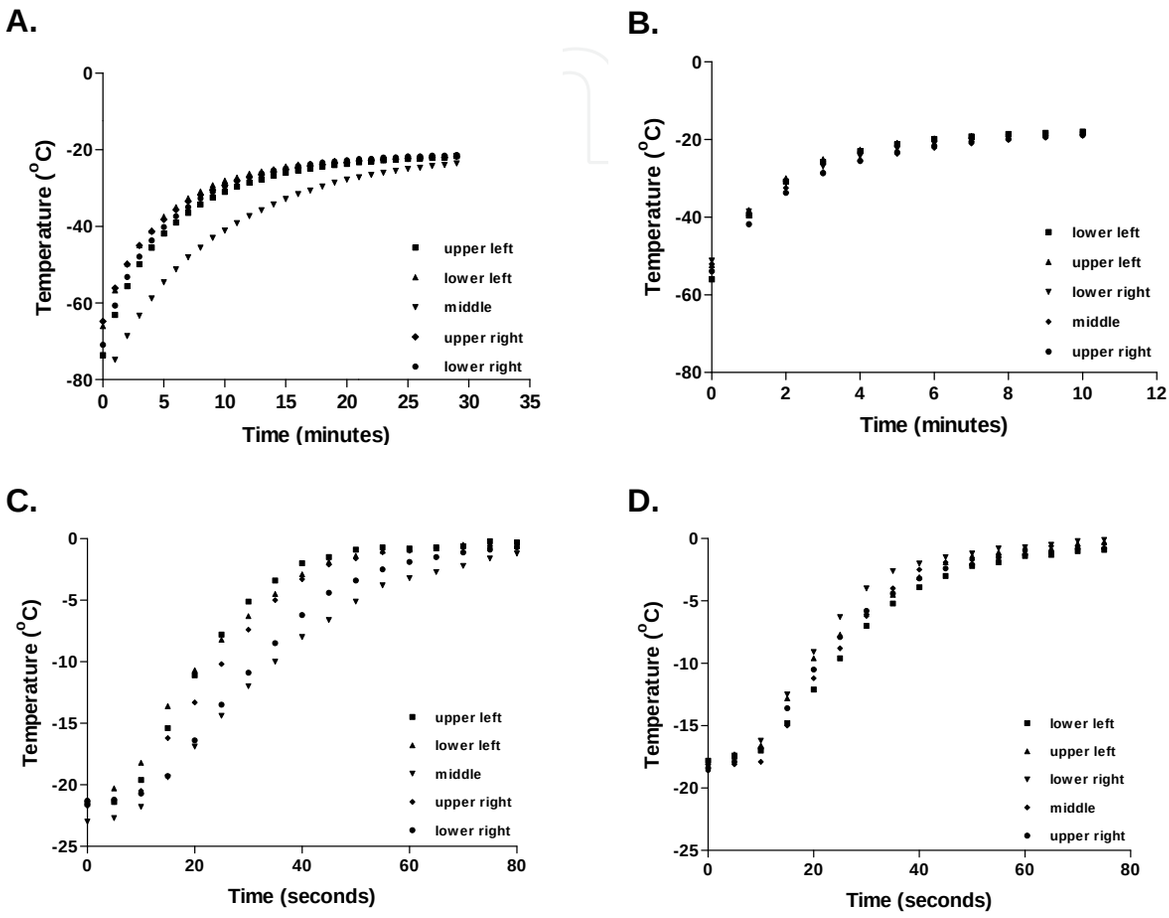


Figure 6. Warming temperature profiles. The wells of a 96-well plate were set up and cooled as described in the text. The plate was then removed from cryostorage and placed in a –20°C freezer until equilibrated before being transferred to 37°C for the second step of the warming protocol. Temperatures were measured every minute while at –20°C and every 5 seconds while at 37°C. Graphs (A) and (C) are without heat sinks while graphs (B) and (D) are done with heat sinks and thermal conducting compound

3.3. Cooling conditions

Having established a protocol that uniformly warms wells across a multiwell plate, attention was focused on defining a uniform cooling profile. Cooling rate is one of several cryobiological variables that can impact cell survival and is usually cell type dependent. Slower cooling rates while discouraging undercooling of the samples and the formation of ice inside the cell do allow for longer exposure times to cryoprotectants and time for osmotic changes to occur that promote cell shrinkage which conceivably have a negative impact on the stability of the cell-substrate interactions. Faster cooling rates reduce exposure to the cryoprotectant and osmotic

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changes to the cell but do allow undercooling to occur and can also allow for the formation of ice inside the cell. A balance between these two extremes has to be optimized in order for maximum cell survival to occur. A system using 96-well plates, presents unique challenges in terms of uniform cooling across the plate as well as providing an optimum cooling rate for a particular cell type. Several cooling rates were examined and included, -0.2°C/min, -0.5°C/min, -1.0°C/min, -3.0°C/min, -5.0°C/min, and -10.0°C/min. Cooling rate measurements for each cooling profile were taken for 3 separate runs. Two different sample volumes were evaluated, 100 µl/well and 50 µl/well. For these experiments, the ambient environment of the microtiter plates was uniformly cooled using a programmable controlled-rate freezer (Planar Kryo 10). Not surprisingly, slower rates, such as -0.2°C/min, provided more uniform cooling as opposed to faster rates like -10.0°C/min (Figure 7). However, increasing the volume of cryoprotectant medium within the wells of the plate did affect the ability of the controlled-rate freezer to cool the samples uniformly.

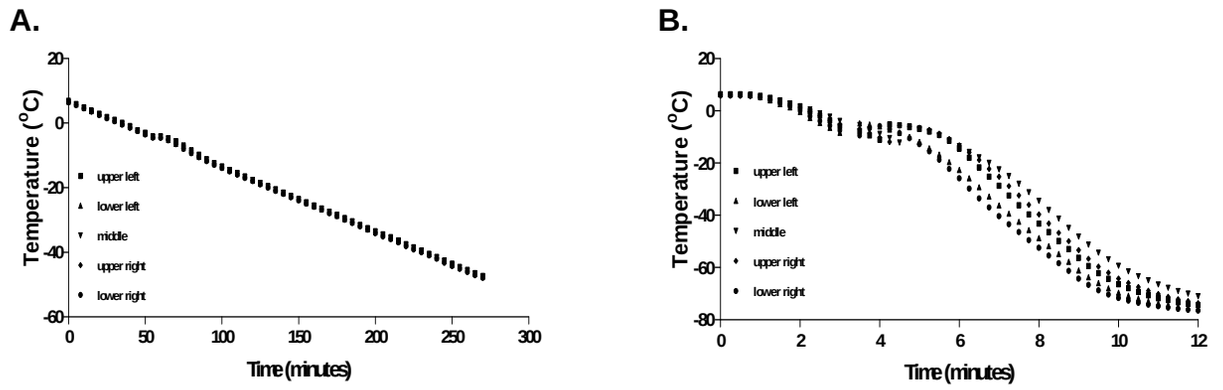


Figure 7. Cooling temperature profiles. All wells of a 96-well microtiter plate (Falcon) were filled with 50 µl of 1M dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS). Thermocouples were inserted into the corner wells and a middle well. The temperature was measured every 5 minutes using a datalogger (Omega) for a rate of –0.2°C/min (A) and every minute for a rate of –10.0°C/min (B).

Cooling profiles at the different locations in the plate were more uniformly controlled when the smaller sample volume was employed (Figure 8). Variations between the well locations were greatest in the region of nucleation when the latent heat of crystallization is evolved (referred to as the latent heat bump in the cooling curve and occurring at approx –5°C in this protocol). This is important because varying degrees of undercooling and the consequential variable cooling rates in the immediate post-nucleation phase are known to influence cell survival [18]. Nucleation is a statistical event that is known to depend upon a number of factors, of which sample volume is critical. For example, cryopreservation of cell suspensions is conventionally carried out in ampoules containing sample volumes of 1 mL or greater. In such cases it is usually observed that spontaneous nucleation occurs at widely different temperatures between ampoules. In other words, there is a variable degree of undercooling between

samples before heterogeneous nucleation is initiated. This undesirable phenomenon has led to the common practice of induced nucleation (so called "seeding") to ensure uniform conditions between replicate samples in a cooling run.

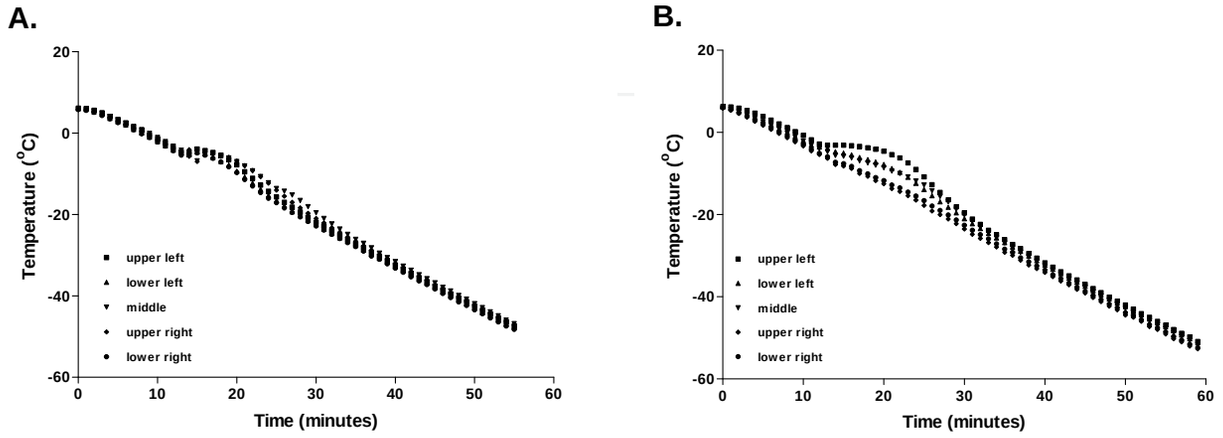


Figure 8. Cooling temperature profiles with different volumes. Plates were set up as described for Figure 1. The plate was cooled at –1.0°C/min to –80°C in a controlled-rate freezer (Planar) and the temperature was measured every minute using a datalogger (Omega). (A) 50 µl/well, (B) 100µl/well.

On the assumption that the five strategic measurement locations in this study are representative of all the wells in a plate, it appears that the spontaneous nucleation of 50µl samples (Figure 8A) is reasonably consistent and more so than the 100µl samples (Figure 8B). Unlike dealing with the freezing of a few ampoules, manual seeding of multiple samples, or simultaneous seeding of all the wells in a microtiter plate, is not practically feasible. Manufacturers of programmed cooling machines have attempted to deal with this by offering the facility for forced nucleation by a momentary sharp drop in ambient temperature initiated by a blast of refrigerant into the cooling chamber. A cooling profile developed in our lab for routine use in screening cryoprotectant solutions on plates that is a modification of a standard –1.0°C/min cooling rate includes a sudden temperature drop to initiate nucleation. It is included here for comparison purposes with regards to conventional cooling profiles, such as –1.0°C/min, and for its potential benefits in terms of cell viability and cell attachment.

From the results presented (Figure 9), it is clear that lower cooling rates provide better cell viability and cell retention ($p<0.001$). For the cells used in these experiments, bovine corneal endothelial cells (BCE), cooling rates above –1.0°C/min demonstrated reduced viability and cell retention. Equivalent cell viability and attachment was observed with cooling rates of –1.0°C/min or less. The modified cooling profile (MP) also demonstrated viability equivalent to a standard –1.0°C/min cooling rate, but better cell attachment after thawing.

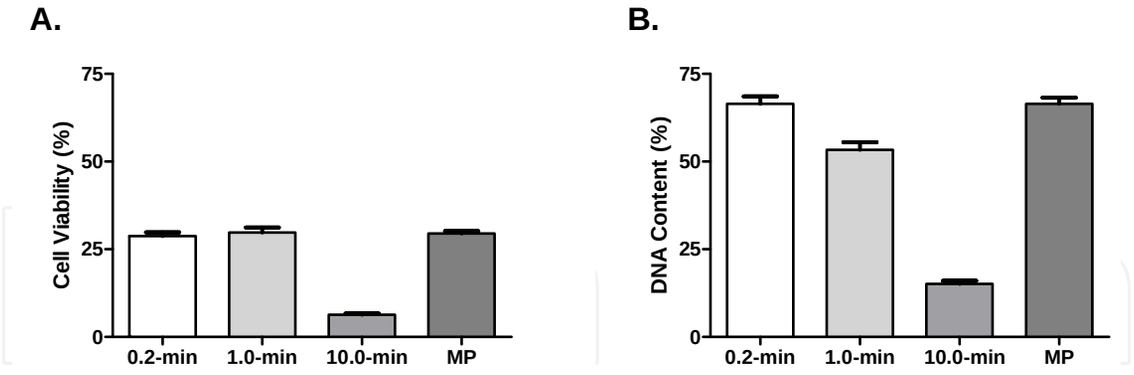


Figure 9. Cell viability and attachment after cryopreservation with varying cooling rates. BCE cells were plated and cryopreserved in 1M DMSO. After thawing metabolic activity (A) and cell attachment (B) via DNA content were measured.

3.4. Cryopreservation in bulk solution vs. air

Another option that was tried was to compare cryopreserving BCE cells in solution versus exposing the cells to CPA and then removing the supernatant to cryopreserve the cells in air. This set of experiments was included on the basis of previous work that showed an improvement in viability of whole corneas when they were cryopreserved in air [19-20]. BCE cells were plated at 20,000 cells/well and cryopreserved in 2M DMSO or 2M 1,2-propanediol (PD). Upon thawing, using the two-step warming protocol, cell viability and cell retention was assessed (Figure 10A). Both DMSO and PD mean cell viability was significantly higher for cells cryopreserved in bulk solution compared with those frozen in air ($p<0.001$). Cell retention determined as DNA content, showed a somewhat similar trend between these two modes of cryopreservation. In the presence of DMSO there was a significant difference between air and bulk medium, with an improvement in cell retention observed when cells were cryopreserved in bulk solution ($p<0.001$). For PD, freezing without bulk medium (in air) resulted in the highest

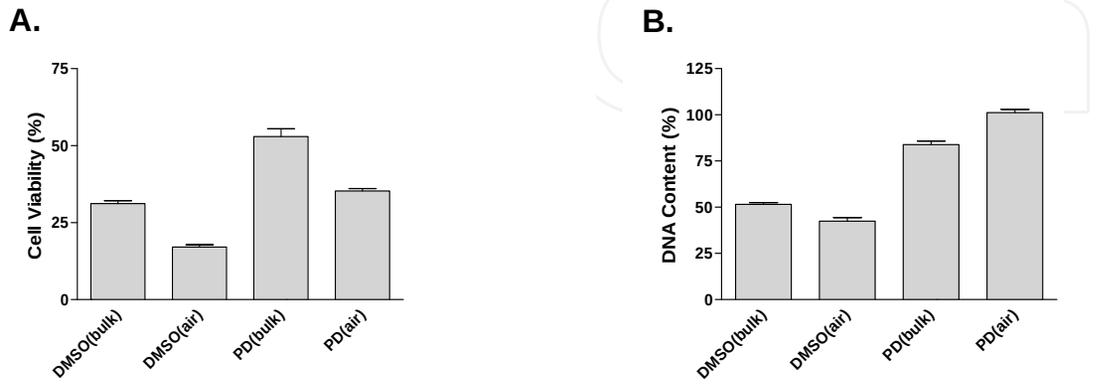


Figure 10. Cryopreservation with and without bulk solution. BCE cells were cryopreserved in 2M DMSO or 2M PD in bulk solution or in air. The graphs for DMSO represent the mean (±SEM) of 252 replicates. Graphs for PD represent the mean (±SEM) of 92 replicates. Statistical analysis done by 2-way ANOVA and presented in the summary.

retention of cells (100%) over freezing in bulk solution ($p<0.001$). It thus appears from these studies that there is a complex relationship between the nature of the CPA and the role of the surrounding medium (liquid or air) in determining both cell retention and cell viability. Moreover, it appears that in this system using BCE cells attached to plastic, a different response is achieved compared with corneal endothelial cells cryopreserved in situ in their native state in whole corneas [20].

3.5. Exogenous nucleating agents

Still another approach was to include an inert nucleating agent within each sample to promote nucleation uniformly at, or just below, the equilibrium freezing point. Such an agent is the commercial product "Snowmax" used effectively in the snow-making industry at ski resorts. We used this product as a tool to compare spontaneous nucleation with enforced nucleation in our system. However, addition of the nucleating agent did not improve the uniformity demonstrated in Figure 7, which was generated in the absence of Snowmax. It is concluded therefore, that slower cooling rates such as -1.0°C/min used with a microtiter plate containing 50μl samples per well yields sufficiently uniform cooling without the need for artificial nucleating techniques.

3.6. Effects on cell survival

Having demonstrated the development of a cooling and warming protocol with satisfactory uniformity across the entire plate, the next step was to examine the effect upon cell survival. To this end, we dispensed BCE cells into each well of a 96-well plate and cryopreserved them in 2M DMSO. The plates were thawed using the modified warming protocol with heat sinks and thermal conduction compound (Figure 11). Recovery of cells and their viability at each location in the plate showed that with the exception of the left edge cell viability was uniform across the entire plate. Interestingly, the left edge of the plate demonstrated viabilities greater than the rest of the plate. Why the other edges do not also demonstrate this effect is unclear. The Cyquant assay measures the DNA content of the cells which is indicative of cell number and is a measure of cell retention on the plate after cryopreservation, was done on the same plates after Alamar Blue. Observation of this data showed that the middle of the plate demonstrated better cell retention than any of the edges.

The data from these experiments was obtained using the modified –1.0°C/min cooling profile (MP) mentioned above that includes a sudden temperature drop to facilitate nucleation. Experiments were also done using other cooling rates such as –0.2°C/min which did not produce more uniform cell viability and cell retention as would be anticipated based on the temperature profile data (see Figure 7). While the temperature profile data showed that a lack of induced nucleation did not affect uniform cooling, nucleation is a statistically random event and does have a significant impact on the survival of the cells being frozen. Based on the cell viability and cell retention data, it is likely that heterogeneous nucleation is taking place even though the temperature profiles of the five wells that were being measured did not indicate it. The data using the modified cooling profile supports this assumption (Figure 9) because it is designed to initiate nucleation across the plate in a limited way and better cell viability was

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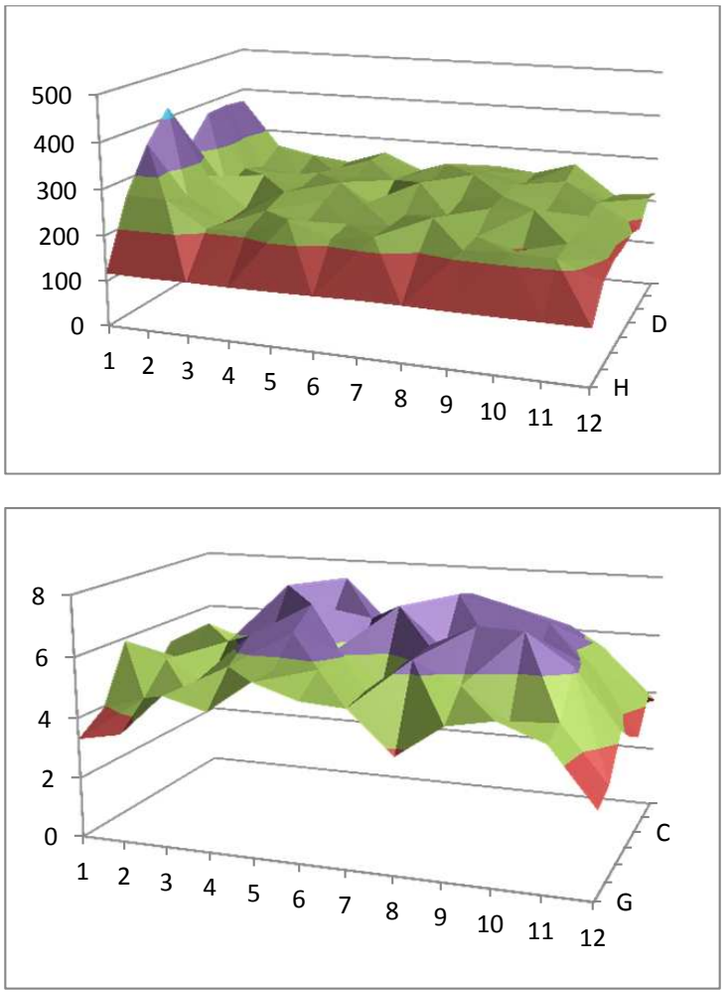


Figure 11. Plate uniformity with cells. BCE cells were plated at $2x10^4$ cells/well and cryopreserved in 2M DMSO. Thawing was done as described in the text. Cell viability (top) and cell retention (bottom) were assayed by Alamar Blue and Cyquant, respectively. The graph represents the layout of a 96-well plate and is the mean relative fluorescence units (RFU) for each well from three experiments. Rows A-G contain cells. Row H represents control wells with no cells and is not shown.

measured when it was used. Therefore, controlled nucleation across the plate is a desirable objective to promote more uniform cell survival.

A system for nucleating all the wells of a 96 well plate was designed. The idea for this system involves using an external liquid nitrogen source. The nucleation manifold consists of spring loaded metal pins, one for each well of a 96 well plate. The plate of pins sits on an aluminum block that allows liquid nitrogen to be pushed through it, thus cooling the block to temperatures well below freezing (~-100°C). Nucleation is achieved by cooling the microtiter plate to a temperature near the freezing point of the solution then pressing the plate onto the spring loaded pins that have been cooled to a much lower temperature. Once nucleation occurs the plate is removed from the pins and the cooling cycle can continue until complete. A representative profile using the manifold and showing nucleation of the corner wells and middle wells of a 96 well plate is presented (Figure 12). Note that each well nucleates within the time span

of ~30-45 seconds and that the variation in the temperature at which nucleation is initiated and the amount of latent heat released is minimal.

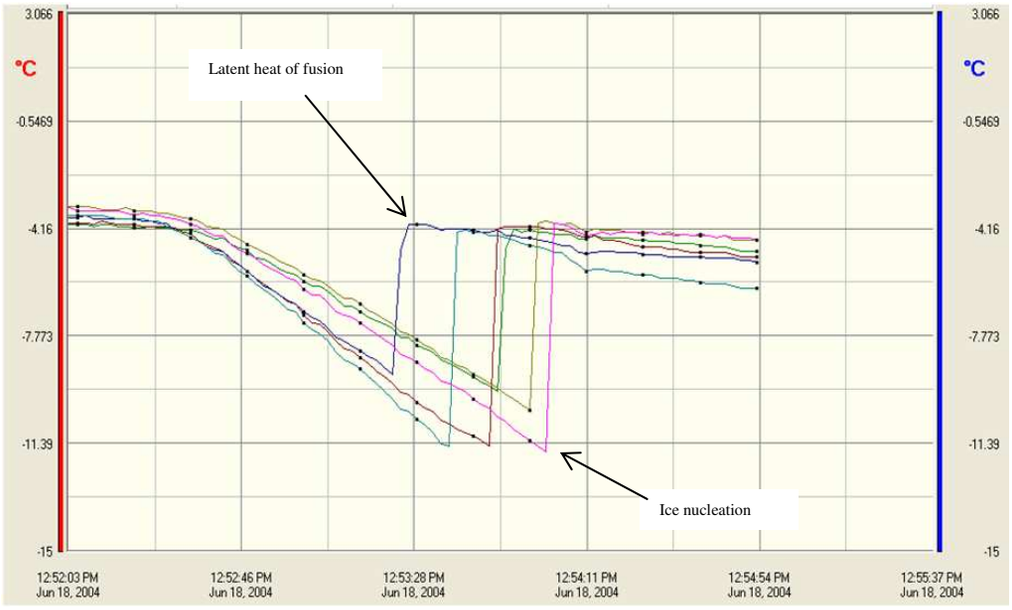


Figure 12. Temperature profile using nucleation manifold. Thermocouples were placed in the corner wells and middle wells of a 96 well plate. Temperatures were recorded every few seconds.

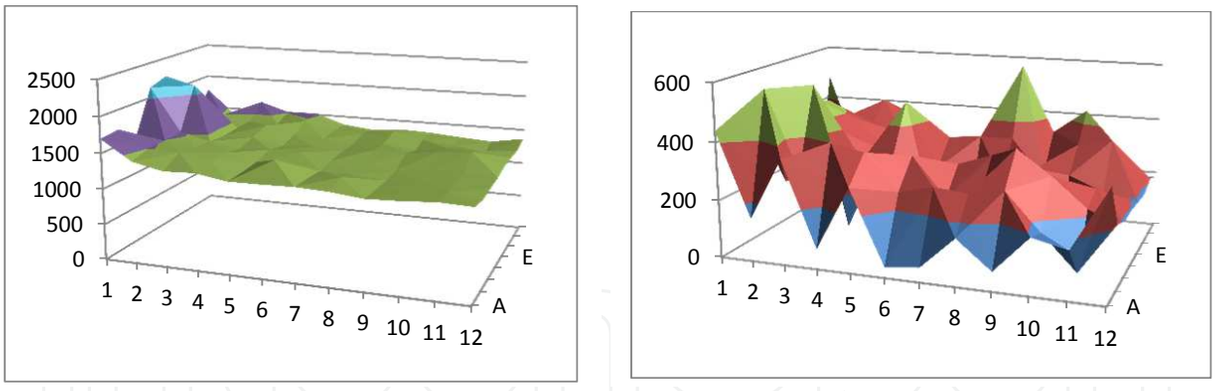


Figure 13. Cell viability and cell attachment of BCE cells after cryopreservation using the nucleation manifold. BCE cells were plated in each well of a 96 well plate and left in 2M PD-EC before being cryopreserved using the nucleation manifold. Viability was measured by alamarBlue™ and DNA content was measured by the Cyquant assay. (left) cell viability. (right) cell attachment.

Experiments were then done to determine whether or not more uniform viability and cell attachment was achieved using this nucleation device. This experiment was performed many times using both DMSO and PD as the CPA. Figure 13 is a representative experiment where viability and cell retention were measured. It was easier to achieve more uniform viability then cell retention. Having done this experiment many times we have noted user variation. Pressure

applied to the plate sitting on the pins is applied strictly by the user using a lever. It has been noted that variations in the handling of the lever produced variations in the uniformity of nucleation across the plate. The experiments to date certainly support the mechanism of nucleation that has been developed, but it is obvious that further optimization of this apparatus is required to improve the uniformity of viability and cell retention across the plate.

4. Other parameters

In natural tissues, or engineered tissue constructs, as well as cell monolayers, that could conceivably be used for cell based assays, maintenance of cell-cell interactions as well as cell-substrate adhesion will be important for full function after cryopreservation. Therefore, the effect of cell confluency on the ability of cells to remain attached to a substrate during cryopreservation was evaluated. Some research groups have suggested that a monolayer reacts differently than subconfluent cell populations and demonstrated a loss of cells upon rewarming. It is hypothesized that because the cells have contacts with each other, when one cell becomes detached it will cause surrounding cells to detach also, even if those surrounding cells are still viable [3]. Other groups agree that monolayers behave differently from subconfluent cell populations when they are cryopreserved, but argue that optimization of cryopreservation variables such as cooling rate can produce cell viability and attachment values consistent with subconfluent cell populations [21]. Various cell densities, both subconfluent and a monolayer, were assayed for cell viability and cell attachment after cryopreservation. BCE cells were plated on tissue culture plastic at varying densities up to a monolayer of cells that consisted of 80,000 cells/well. After exposure to 2M DMSO, the plates were cryopreserved at one of two cooling rates, either -1.0°C/min or with the modified -1.0°C/min cooling profile (MP). The results presented in Figure 14 demonstrated similar cell viabilities regardless of the cell density assayed. More confluent cell populations showed better cell retention compared with more sparsely populated wells (i.e: 2,500 cells/well vs 20,000 cells/well). Slower cooling rates were also evaluated (data not shown) with similar results. It can be concluded, that in this system, the degree of confluency does not greatly impact cell viability and cell retention after cryopreservation. However, different cell types may behave differently when cryopreserved as a monolayer. In addition, the previous studies cited used glass cover slips and microtiter plate filter inserts to study the cryopreservation of a monolayer while viability and cell retention were evaluated on tissue culture plastic in the present study.

In a related experiment, BCE cells were cryopreserved in two different cryopreservation solutions, 2M DMSO and 2M PD, at a subconfluent density of 20,000 cells/well and as a monolayer at 80,000 cells/well (Figure 15). No significant differences in viability or cell attachment were observed. While initial viability was only 50%, the cells were able to initiate proliferation within 24 hours post thaw and continued to proliferate at 4 days post thaw. Cell attachment was very good with values that were 75% or greater based on an untreated control demonstrating that the monolayer was just as robust as the subconfluent population after cryopreservation.

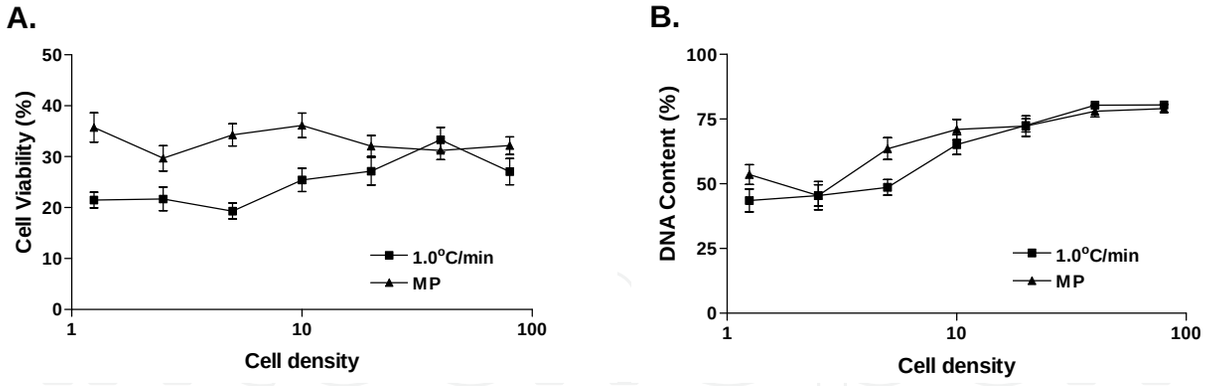


Figure 14. Cell viability and attachment after cryopreservation for varying cell densities. BCE cells were plated from 0-80,000 cells/well and cryopreserved as indicated in the text. Cell viability (A) and cell retention (B) were assessed and the data represent the mean (±SEM) of 12 replicates. Cell density is expressed using a log scale.

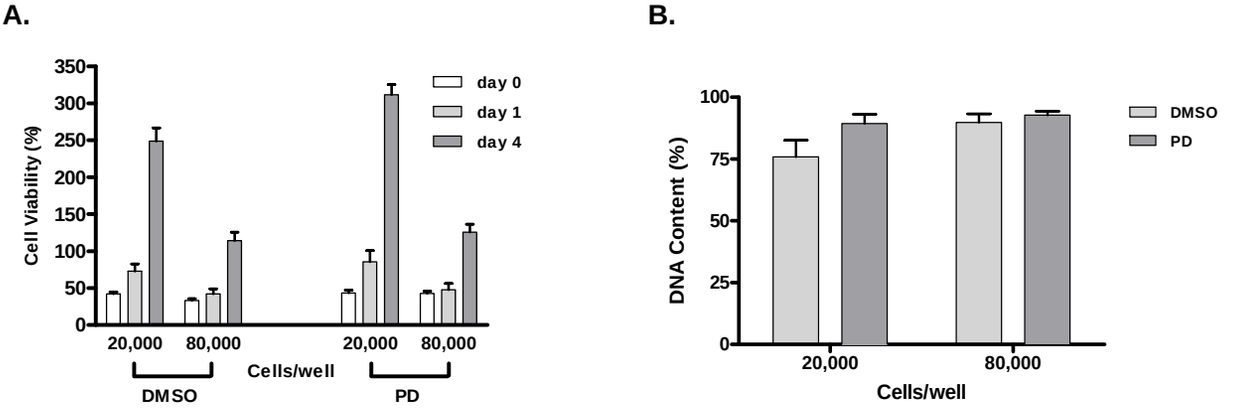


Figure 15. Percent cell viability and DNA content of a subconfluent cell population and a cell monolayer after cryopreservation. BCE cells were cryopreserved in 2M DMSO in HBSI or 2M PD in EC at the indicated cell densities. Viability was measured by alamarBlue™ and DNA content was measured by the Cyquant assay (Molecular Probes). Percent viability and DNA content is the mean (±SEM) of 10 replicates.

4.1. Optimized system

Having optimized the cooling and warming protocols for cryopreserving cells in a multiwell plate, experiments were then done to evaluate the system and how well it could be used to measure cell viability in cryopreservation experiments. Two commonly used cryoprotectants, DMSO and PD were assessed in Euro-Collins (EC), a well established vehicle solution used in cryopreservation, at concentrations of 0-4M. BCE cells were plated at a density of 20,000 cells/well and exposed to DMSO and PD before being cryopreserved using the modified –1.0°C/min protocol. The graphs depict cell retention for both CPA and their viability after cryopreservation (Figure16). For both CPAs, concentrations of 1-2M demonstrated the best cell viability and attachment. Higher CPA concentrations showed viability and attachment that was significantly worse ($p<0.01$). At a concentration of 2M PD, cell viability of ~77% was observed versus only ~50% for the same concentration of DMSO. For both cell viability and

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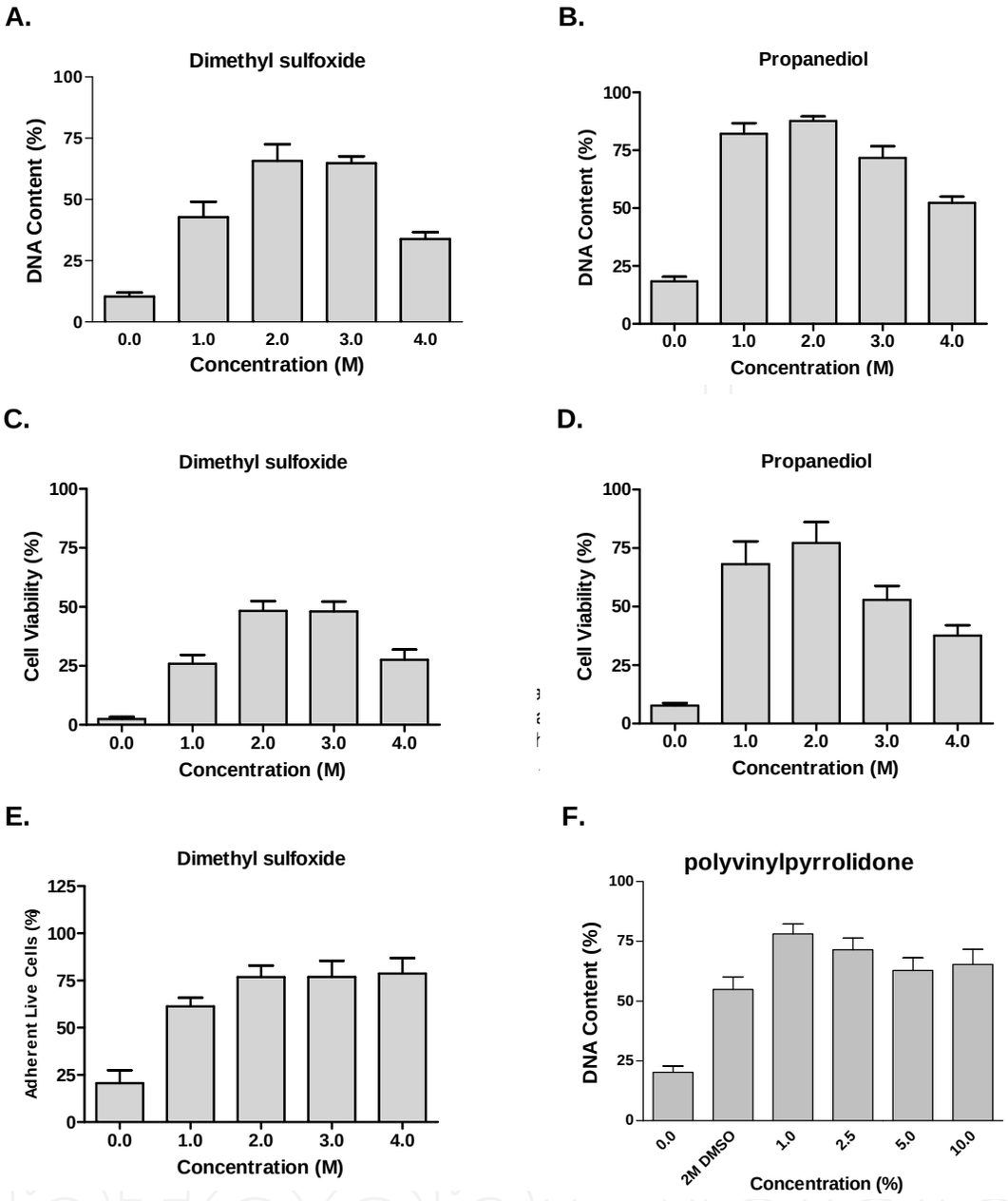


Figure 16. Cell viability and retention after cryopreservation. BCE cells were plated and cryopreserved as described in the text using the modified –1.0°C/min cooling profile. Graphs A & B show DNA content which is indicative of cell retention. Graphs C & D show cell viability. Graphs E & F show viable adherent cells. All graphs represent the mean (±SEM) of 12 replicates. Statistical analysis done by 2-way ANOVA and presented in the summary.

cell retention after cryopreservation, PD demonstrated better values over DMSO at all concentrations evaluated ($p<0.001$). DNA content values of >75% were achieved using 2M PD in EC over the same concentration of DMSO which achieved values of only ~60-65%. Taking this a step further, an evaluation of the viability of those cells still attached to the plate was done, expressed as adherent live cells (Fig. 16 E and F). It was observed that adherent cells were >60% live (viable) for all concentrations of DMSO tested and >70% for all concentrations of PD. So, cells that remained attached after cryopreservation had a high level of viability at

optimal CPA concentrations. Further study of other cryoprotectants could yield even better viability and cell attachment values. In our experience to date, the CryoPlate™ protocol works for most cells we have evaluated but the optimal CPA requirement varies.

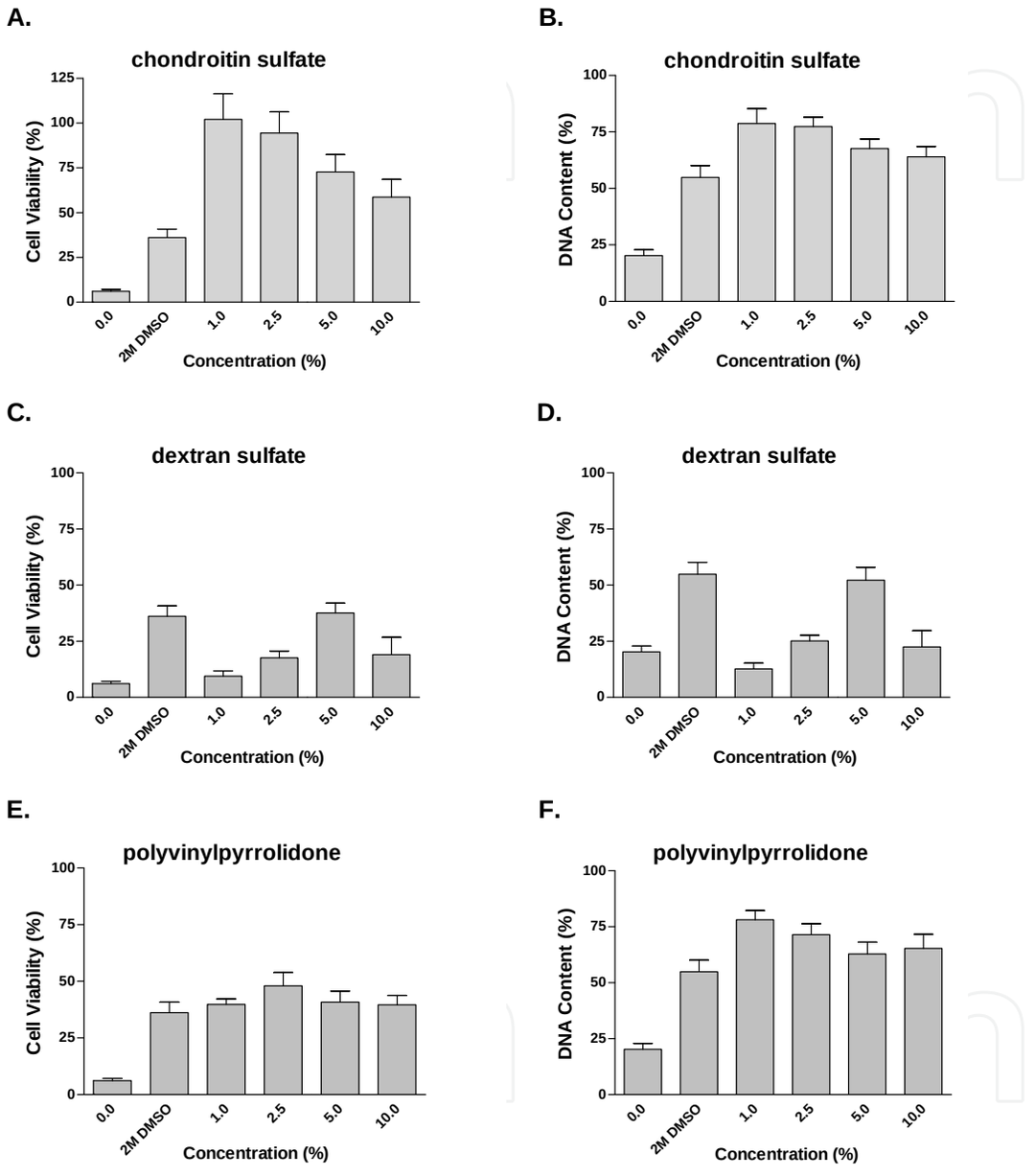


Figure 17. Cell viability and retention after cryopreservation using macromolecular CPAs. BCE cells were plated at 20,000 cells/wells, exposed to the above compounds at the indicated concentrations with 2M DMSO in EC and cooled using the modified –1.0°C/min cooling program. Graphs A, C & E show cell viability and graphs B, D & F show DNA content. All graphs represent the mean (±SEM) of 12 replicates. Statistical analysis done by 2-way ANOVA.

In a second series of experiments, non-permeating macromolecular cryoprotectants were also examined in combination with the cell permeating cryoprotectants DMSO and PD. Chondroitin sulfate (CS), dextran sulfate (DS) and polyvinylpyrrolidone (PVP) were assayed at various

concentrations (0-10%) in the presence of 2M DMSO for their ability to enhance viability and cell retention. Concentrations higher than 10% for each of the CPAs suggested proved to be difficult to get into solution above 10%, hence this defined the upper limit of the concentrations tested. BCE cells were assayed for cell viability and DNA content as described above and the results are presented in Figure 17. Using 2M DMSO as a baseline, which demonstrated a cell viability of ~40% with a DNA content of ~55%, enhancement of viability ($p<0.001$) and cell retention ($p<0.05$) was observed with CS. A concentration of 1% CS in 2M DMSO demonstrated cell retention at ~100% with viability at ~75%. Dextran sulfate did not improve viability or cell retention over 2M DMSO alone. At the best concentration of dextran sulfate (5%), viability and cell retention were equivalent to using just 2M DMSO. Cell retention was improved over 2M DMSO alone with all concentrations of PVP examined ($p<0.05$) but only a slight improvement in cell viability at a concentration of 2.5% PVP. In summary the only macromolecular cryoprotectant that demonstrated benefits for cells with regards to cryopreservation was chondroitin sulfate. In fact this combination provided results that were not significantly different compared with untreated controls. Chondroitin sulfate is readily found in association with the extracellular matrix of cells and this may explain why greater viability and cell retention were observed compared with the other macromolecular CPAs that were evaluated.

4.2. Carrier solution

Different carrier solutions were evaluated to determine if they had an impact on cell viability during cryopreservation. Others have reported the significance of the cryoprotectant carrier solution to overall viability after preservation [22-24]. In this experiment, four carrier solutions were used, Dulbecco's Modified Eagles Medium (DMEM), Euro-Collins (EC), a well established organ preservation solution, Hepes-buffered saline (HBSI), and Unisol (UHK), a preservation formulation developed in our labs. BCE cells were exposed to 2M DMSO in the carrier solutions listed and then cryopreserved using the optimized cooling and warming conditions described above, Cell viability was assessed by measuring metabolic activity immediately after thawing and for several days post thaw. The best viability was observed when cells were cryopreserved in either HBSI or UHK. Viability was as high as 70% and there was evidence of recovery and proliferation by day 3 post thaw with an increase in viability close to 100% of untreated controls. The least viable cells were observed while cryopreserved in EC and their recovery post thaw was also minimal. Using this system, it was observed that the choice of carrier solution can have a significant impact on cell survival. It is likely that different vehicle solutions may combine effectively with other CPAs.

4.3. Apoptosis

Using our system for cryopreserving cells on plates, apoptosis was evaluated. The nexin staining assay was performed and measured using the Guava Cell Analysis System on cells that had been cryopreserved as a monolayer and as a subconfluent cell population in 2M DMSO or 2M 1,2-propanediol (Table 1). Apoptosis was evaluated up to 2 days post thaw to determine if apoptosis was occurring. Immediately after thawing, there was little if any evidence for apoptosis. At day 1 post thaw, some apoptosis was occurring so that the percent

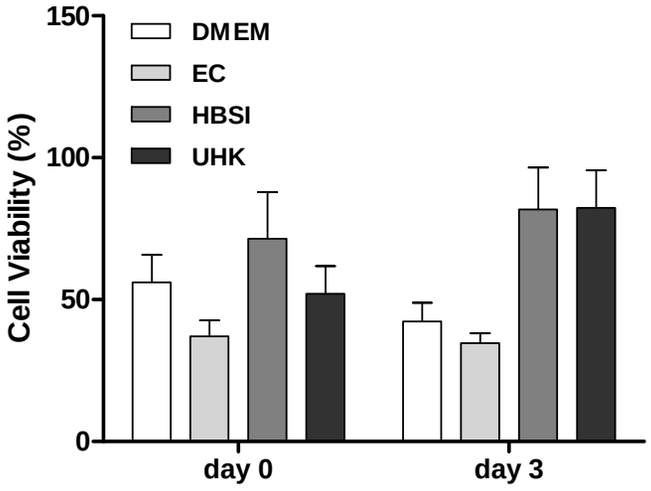


Figure 18. Cell viability of BCE cells after cryopreservation in different carrier solutions. Cells were cryopreserved in 2M DMSO in the indicated carrier solutions. Percent cell viability is the mean (±SEM) of 12 replicates

of viable cells had dropped to between 80-90%. However by day 2 apoptosis was detectable at very low levels if at all.

Cell density	CPA	Day 0		Day 1		Day 2	
		Viable†	Apoptotic*	Viable†	Apoptotic*	Viable†	Apoptotic*
20,000 cells/ well	DMSO	97.9	2.1	80.8	19.2	99.3	0.7
	PD	94.2	5.8	82.7	17.3	95.5	4.5
80,000 cells/ well	DMSO	98.3	1.7	91.9	8.1	99.8	0.2
	PD	97.7	2.3	88.5	11.5	98.9	1.1

† Viable cells are calculated as a percentage of the total cells counted: average number of cells counted is 1000

*Apoptotic values include early and late apoptosis events and any resulting dead cells

Table 1. Apoptosis in BCE cells after cryopreservation

4.4. Well configurations

In addition to the 96 well plate configuration, four other different well configurations were evaluated for their ability to produce viable cells after cryopreservation using the cooling and warming protocols established. As a first step, temperature profiles were measured for each configuration. Six thermocouples were placed into wells at each corner of the plate and in two wells at the middle of the plate. For the 6-well plate, a thermocouple was placed in each well. Temperature profiles were measured every minute using a datalogger (Omega) during a cooling profile of -1.0°C/minute in a controlled rate freezer (Planar). While it was anticipated that for most of the cooling regime, the plate would follow the same cooling pattern as the freezer, part of this process was to determine when the latent heat of fusion occurs, when water changes to ice, and how significant the release of heat was. Using this information, a modified protocol can be developed that would help alleviate the latent heat of fusion or at least reduce

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it to a level that causes minimal disruption to the cooling profile of the sample. In addition to the 6-well and 384-well plates (Figure 18), 12-well and 24-well configurations were also evaluated. Each well configuration demonstrated varying degrees of latent heat. The 384-well demonstrated the most variable release of latent heat while the 6-well plate release of heat did not appear to be as abrupt and was not as distinct as for the other well configurations.

This did not mean that the cooling profile for the 6-well plate was actually better, in fact, when viability was measure for each of the well configurations (Table 2), the 6-well plate demonstrated the lowest cell viability and cell attachment. Cell attachment for all the well configurations except the 6-well plate demonstrated attachment values of at least 90% or greater as compared with an untreated control. Viability was much more variable and was indicative of the degree of latent heat observed during the recording of the temperature profiles for each well configuration. Surprisingly, the 384-well plate demonstrated cell viability that was as good as that observed with the 96-well plate. Every well of the 384-well plate was not evaluated during these experiments and so while these results demonstrated that high viability values are achievable, there is still may be an issue with uniform viability and attachment in every well of a plate regardless of the well number and configuration.

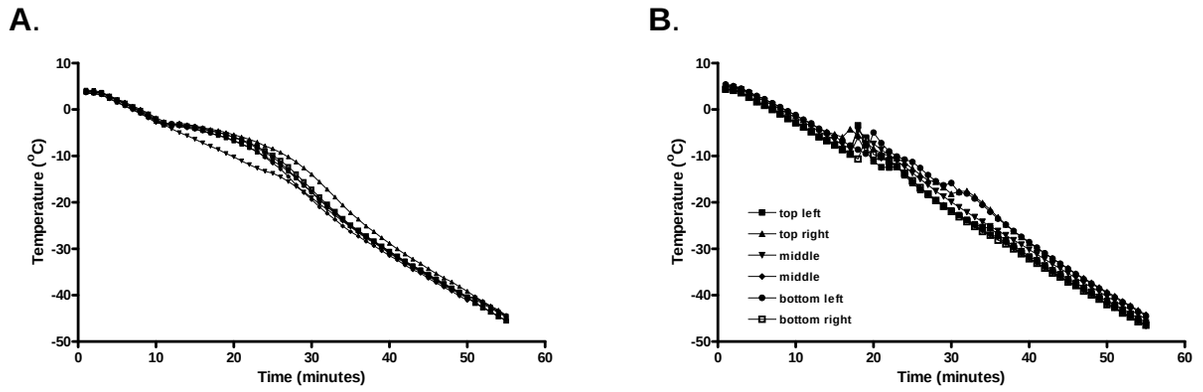


Figure 19. Temperature profiles for a 6-well and a 384-well microtiter plate. Thermocouples were placed into the corner wells and middle wells of each plate. Temperatures were measured every minute with a datalogger during a cooling profile described in the text. The graphs represent one of several experiments performed for each well configuration. (A) 6-well. (B) 384-well.

Cell Viability %			Cell Attachment %	
Well configuration	**Day 0**	**Day 3/5**	**Well configuration**	**Day 0**
6-well	25.845±1.04	127.81±6.66	6-well	43.12±8.79
12-well	24.53±1.20	125.25±3.72	12-well	90.32±3.93
24-well	43.73±1.50	185.35±6.60	24-well	94.66±3.05
96-well	71.52±16.28	81.76±14.84*	96-well	89.76±3.57
384-well	72.62±5.50	169.02±7.46	384-well	125.61±2.08

*Only the 96-well configuration viability is from day 3. All others are from day 5.

Table 2. Cell viability and attachment with differing well configurations

5. Discussion

In this chapter, development of a method for cryopreserving cells on a fixed substrate has been described (US Patent #6,596,531) [17]. Specifically, a CryoPlate™ protocol has been developed to freeze cells on microtiter plates. This protocol was developed in our labs as a method for the rapid screening of cryoprotectant solutions. However, its application for other cell-based assays is far reaching. Potentially, any adherent cell can be cryopreserved using this system for a variety of applications upon thawing.

Initial experiments using a traditional approach for cryopreserving cells in suspension that involved slow rate cooling and rapid thawing at 37°C produced no viable cells. It was noticed that as the polystyrene plastic plate cooled, the plastic constricted and the plate contorted or bowed at its edges. It was also noticed during the initial experiments that the cells came off the plate. One can speculate that the sudden change in temperature causes stresses resulting in the plastic changing shape, similar to what occurs when an ice cube is placed in a warm liquid and it develops cracks. The sudden temperature change and abrupt changes to the plastic culture plate as it warms causes the cells to be forcefully detached and this detachment may trigger the death of the cell via apoptosis or other cell death mechanisms. Instead, a two step warming protocol was tried in which the plates were warmed to an intermediate temperature before being rapidly thawed at 37°C. The idea being a more gradual warming would allow the plate to readjust to warmer temperatures without abrupt changes in its shape which would prevent the cells from coming off during warming, promoting greater cell viability and attachment after thawing. The first protocol involved removing the plates from cryostorage and leaving them in air (~25°C) until they reached ~-20°C before undergoing rapid thawing at 37°C. Plates were then placed on ice for cryoprotectant removal and subsequent assessment of viability. Using this protocol, viable cells were obtained after freezing.

Focus then shifted to applying these conditions across an entire plate. Preliminary measurements of temperature at strategic locations within the plate demonstrated differential warming profiles. Uniform warming was achieved by removing the plate from cryostorage and leaving the plate in a -20°C freezer for 30 minutes followed by rapid thawing at 37°C using an aluminum heat sink with thermal conducting compound prewarmed to 37°C [17]. As mentioned previously, the intermediate incubation at -20°C allowed the plate to warm enough to relax the plastic so that no distortion remained and also allowed all the wells to equilibrate to a similar temperature before the rapid thawing facilitating uniform warming conditions. As was observed in Figure 6, uniform warming conditions were achieved.

Uniform cooling was also sought. In particular, synchronizing ice nucleation in each well was a primary objective as this would insure that all the wells of a plate had a similar cooling profile which should in turn produce similar cell viability upon thawing. A survey of various cooling rates demonstrated that slower cooling rates were better at achieving similar profiles across wells including more synchronous nucleation than faster cooling

rates. It was also noted that the volume of solution in the wells impacted the cooling profiles of measured wells. More similar cooling profiles were possible when smaller solution volumes were used. However, completely removing all solution once the cells had been exposed to cryoprotectants prior to freezing did not improve viability and in fact impacted cell viability negatively. Nucleation is a random event and so further steps were taken to control nucleation so that all wells of the plate were nucleating together. The use of an exogenous nucleating agent, such as Snomax, was investigated and did not improve ice nucleation uniformity. A modification to the -1.0°C/minute cooling profile was made that included a sudden drop in temperature which promoted more uniform ice nucleation across the plate. This improved the consistency of viability and cell retention across the plate, but some variations still existed predominantly along the left edge of the plate.

Efforts were then made to further improve synchronous ice nucleation across the plate by the application of a nucleation manifold. Described in the text, this device was designed to promote ice nucleation across the plate in a controlled and timed manner. Further improvement in uniform viability was observed, but uniformity of cell retention did not show any significant improvement. As the nucleation manifold was considered a prototype, further improvements are envisioned that would automate the process and remove variation cause by user inconsistencies. There is also the possibility that cell retention is actually improved as well and that the decreased uniformity of cell retention has to do with inconsistent cell plating prior to cryopreservation. Further experiments are in progress to answer these questions and promote more uniform cell retention that is consistent with cell viability values.

In addition to cooling and warming, other parameters were considered that might affect the viability of cells attached to a plate. Cell confluency was felt to play an important role in viability and retention of cells on a fixed substrate with the thought that subconfluent populations would have better retention than a monolayer of cells. Monolayers have developed cell to cell connections similar to cell-cell and cell-matrix connections that are present in tissues. It was hypothesized that when one cell detached that would cause neighboring cells that had developed connections to the detaching cell to come off the plate as well [21]. Experiments done using our two step warming protocol demonstrated no difference between confluent and subconfluent cell populations. Factors that promoted the detachment of the cells using a one step thawing regime have been alleviated using a two step protocol so that confluency does not have the same influence on outcomes as noted previously by other groups [3]. Maintenance of a monolayer and its cell-cell interactions strongly suggests that our protocol could readily be applied to tissue constructs such as skin equivalents or other types of three dimensional constructs that would be used for drug testing and other applications.

Taking this a step further, nexin staining was performed on subconfluent cells and a monolayer for several days after cryopreservation and thawing on plates to check for the induction of apoptosis. Viability values remained high, ~95% of the counted population, even after 2 days in culture following thawing and no differences were observed between subconfluent and monolayer cell populations. Apoptosis induction was minimal demonstrating that our process promotes cell viability and that the cells are able to function and are available for further assays.

Application of our cryopreservation protocol to microtiter plates of differing well configurations showed variation of cell viability and cell retention that was not necessarily based on the consistency of their cooling profiles. For example, while the 6 well plate demonstrated a relatively consistent thermal profile across its wells, cell viability and cell retention were not as good at 25% and 43% respectively. However, the best viability was observed using the higher well configurations, 96 well and 384 well with cell viability at ~70% and cell retention at >90% even though their cooling profiles were not as consistent or uniform as that observed in the 6 well plate. Other factors are likely involved that influence cell viability during cryopreservation in plates. The volume of the wells is one factor as was observed when thermal profiles were compared using 50µl and 100µl in a 96 well plate. Smaller volumes produced better thermal profiles. With regards to the 6 well plate, the larger volume in each well means it is easier to cool each well consistently particularly at slower rates. However, at faster rates a larger volume is harder to cool or rewarm while keeping the conditions consistent across each well. This is likely why viability and cell attachment values are lower in a 6 well plate versus a 96 well plate. Modifications to the two step warming protocol might be needed to accommodate the changes in volume as the wells get larger so that better viability and cell retention can be maintained.

The initial purpose behind the development of this protocol for cryopreserving cells on plates was to develop a higher throughput system for screening possible cryoprotectant formulations. An example CryoPlate™ protocol, based on the studies presented here, is illustrated in Figure 20. The plates are placed on ice and cryoprotectant(s) added either in a single step or multiple steps of increasing concentration. When the final cryoprotectant solution has been added it is incubated for 10 minutes on ice. Then it is placed in a precooled (4°C) control freezer and once the temperature has returned to 4°C it is cooled at -1°C/min. rate to -80°C with nucleation at -6°C. Then the plates are stored at ≤ –135°C in a vapor phase nitrogen or mechanical storage freezer. The plates are thawed in at least two steps. First by placing in a –20°C freezer for 30 minutes and second, by rapid warming, ~20°C/min. to 0°C, at 37°C. Rapid thawing has been performed using aluminum heat sinks and thermal conduction compound as well as a waterbath, both prewarmed to 37°C with similar results. This warming method minimizes detachment during thawing (Figure 2). The cryoprotectant solution is then removed in one or more washing steps and the plates are incubated for at least 1 hour at 37°C in cell culture medium in a tissue culture incubator to allow cell recovery to occur. We usually utilize rewarmed cell cultures after completion of a 1 hour recovery period.

A survey of several cryoprotectants, penetrating and non-penetrating, and cryoprotectant carrier solutions was done (Figure 16) that demonstrated the utility and versatility of our system allowing rapid selection of different cryoprotectants and carrier solutions as well as their concentration with regards to the the specific cell type assessed. In this particular case excellent cell viability was obtained combining 2 M DMSO and 1% (w/v) chondroitin sulfate in EC solution (Figure 16A). We have since employed the CryoPlate™ method to cryopreserve adherent endothelial cells with trehalose [15] and endothelial cells, smooth muscle cells and heart valve leaflet myofibroblasts using either DMSO or PD formulations with acceptable high

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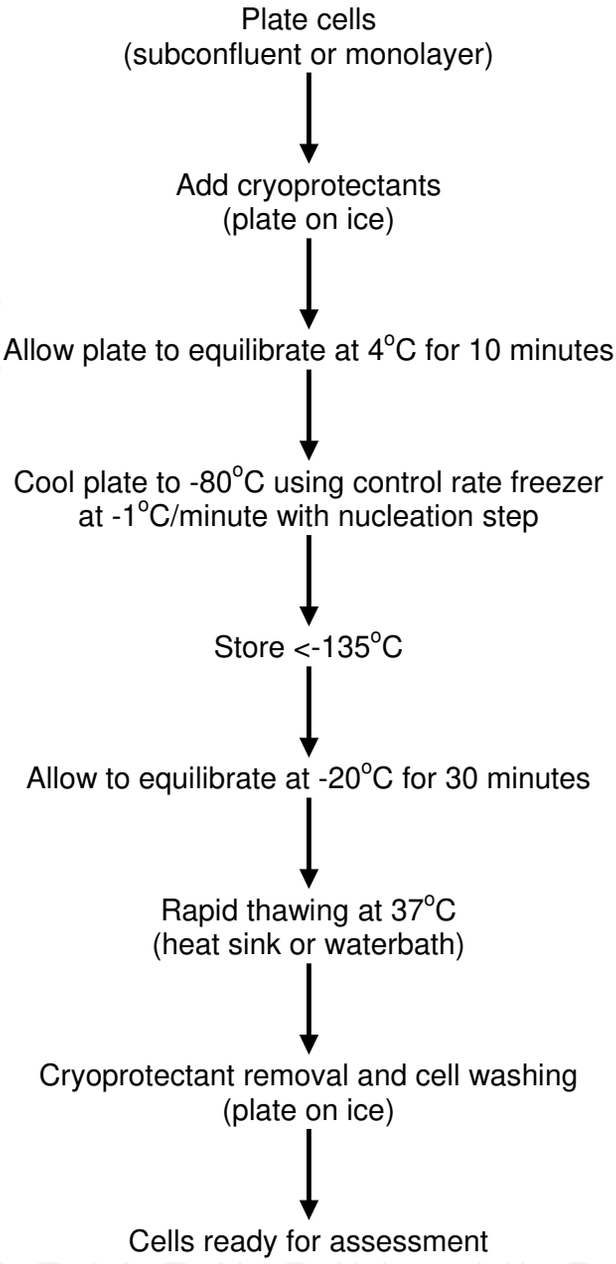


Figure 20. Example CryoPlate™ protocol for cryopreserving cells on microtiter plates.

cell viabilities in combination with other carrier solutions [25-26]. Preliminary results also indicate that our protocol is effective for preservation of human mesenchymal stem cells [28].

Based upon our experience to date, we anticipate that our CryoPlate™ protocol in combination with various CPAs and carrier solutions can be adapted for many, if not all, cell-based assay systems, and possibly tissue constructs. Cell-based assay systems and tissue constructs are currently replacing animal testing, so methods that can preserve and facilitate the accessibility of these types of systems are needed. Our protocol cryopreserving attached cells on microtiter plates fulfills this need and is versatile so that it can be applied to a wide variety of cell types for different applications.

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References

[1] Taylor DL, Woo ES, Giuliano KA. Real-time Molecular and Cellular Analysis: The New Frontier of Drug Discovery. Current Opinion in Biotechnology 2001; 12 75-81.

[2] Hetzel FW, Kruuv J, McGann LE, Frey HE. Exposure of Mammalian Cells to Physical Damage: Effect of the State of Adhesion on Colony-forming Potential. Cryobiology 1973; 10 206-11.

[3] Hornung J, Muller T, Fuhr G. Cryopreservation of Anchorage-Dependent Mammalian Cells Fixed to Structured Glass and Silicon Substrates. Cryobiology 1996; 33 260-70.

[4] McGann LE, Yang H, Huang Z, Penko A, Larese A. Cell-to-Cell and Cell-to-Surface Interactions Affect Responses During Cryopreservation. Transfusion 1993; 33(7) 611.

[5] Ohno T. A Simple Method for In Situ Freezing of Anchorage-Dependent Cells. In: A Doyle, JB Griffiths, DG Newell (eds.) Cell and Tissue Culture: Laboratory Procedures. Chichester: John Wiley and Sons; 1994.

[6] Ji L, de Pablo JJ, Palecek SP. Cryopreservation of Adherent Human Embryonic Stem Cells. Biotechnology and Bioengineering 2004; 88(3) 299-312.

[7] Katkov II, Kan NG, Cimadamore F, Nelson B, Snyder EY, Terskikh AV. DMSO-Free Programmed Cryopreservation of Fully Dissociated and Adherent Human Induced Pluripotent Stem Cells. Stem Cells International 2011; 2011:981606. doi: 10.4061/2011/981606. Epub 2011 Jun 1.

[8] Malpique R, Ehrhart F, Katsen-Globa A, Zimmermann H, Alves PM. Cryopreservation of Adherent Cells: Strategies to Improve Cell Viability and Function after Thawing. Tissue Engineering Part C Methods 2009; 15(3) 373-86.

[9] Xu X, Liu Y, Cui Z, Wei Y, Zhang L. Effects of Osmotic and Cold Shock on Adherent Human Mesenchymal Stem Cells During Cryopreservation. Journal of Biotechnology 2012; 162(2-3) 224-31.

[10] Karow AM. Biophysical and Chemical Considerations in Cryopreservation. In: Karow AM, Pegg DE (eds.) Organ Preservation for Transplantation.New York: Dekker; 1981. p 113.

[11] Mazur P. Freezing of living cells: mechanisms and implications. American Journal of Physiolology 1984; 247 125.

[12] Taylor MJ. Sub-zero Preservation and the Prospect of Long-term Storage of Multicellular Tissues and Organs. In: RY Calne (ed.) Transplantation Immunology: Clinical and Experimental. Oxford, New York, Tokyo: Oxford University Press; 1984. pp. 360-90.

[13] O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (Resazurin) Fluorescent Dye for the Assessment of Mammalian Cell Cytotoxicity. European Journal of Biochemistry 2000; 267 5421–5426.

[14] Campbell LH, Brockbank KGM. Cryopreservation of Adherent Smooth Muscle and Endothelial Cells with Disaccharides. In: Katkov I. (ed.) Current Frontiers in Cryopreservation. Croatia: InTech,; 2012.

[15] Campbell LH, Brockbank KGM. Culturing with Trehalose Produces Viable Endothelial Cells after Cryopreservation. Cryobiology 2012; 64 240-4.

[16] Campbell LH, Taylor MJ, Brockbank KGM. Development of Pancreas Storage Solutions: Initial Screening of Cytoprotective Supplements for β-cell Survival and Metabolic Status after Hypothermic Storage. Biopreservation and Biobanking 2013; 11(1) 12-18.

[17] Campbell LH, Taylor MJ, Brockbank KGM. U.S. Patent #6,596,531. Two stage method for thawing cryopreserved cells. July, 2003.

[18] Diller KR. The Influence of Controlled Ice Nucleation on Regulating the Thermal History during Freezing. Cryobiology 1985; 22 268-281.

[19] Taylor MJ, Hunt CJ, Sherrard ES. Assessment of Corneal Endothelial Integrity by Specular Microscopy after Cryopreservation. In: The Cornea in Health and Disease: Proceedings of VIth Congress of the European Society of Ophthalmology. Royal Society of Medicine international Congress and Symposium Series.1981; 40 437-44.

[20] Fong LP, Hunt CJ, Taylor MJ, Pegg DE. Cryopreservation of Rabbit Corneas: Assessment by Microscopy and Transplantation. British Journal of Ophthalmology 1986; 70 751-60.

[21] Armitage WJ, Juss BK. The Influence of Cooling Rate on Survival of Frozen Cells Differs in Monolayers and in Suspensions. Cryo-Letters 1996; 17 213-18.

[22] Taylor MJ, Campbell LH, Rutledge RN, Brockbank KGM. Comparison of Unisol with Euro-Collins Solution as a Vehicle Solution for Cryoprotectants. Transplantation Proceedings 2001; 33 677-79.

[23] Baust JM, Van Buskirk RG, Baust JG. Improved Cryopreservation Outcome is Facilitated by an Intracellular-Type medium and Inhibition of Apoptosis. Journal of the Biomedical Engineering Society 1999; 1 206-7.

[24] Baust JM, Van Buskirk RG, Baust JG. Cell Viability Improves Following Inhibition of Cryopreservation-Induced Apoptosis. In Vitro Cellular and Developmental Biology 2000; 36(4) 262-70.

[25] Campbell LH, Brockbank KGM. Serum-Free Solutions for Cryopreservation of Cells. In Vitro Cellular & Developmental Biology-Animal 2007; 43 269–275.

[26] Campbell LH, Brockbank KGM. Cryopreservation of Porcine Aortic Heart Valve Leaflet-derived Myofibroblasts. Bioprocessing and Biobanking 2010; 8(4) 211-217.

[27] Campbell LH, Brockbank KGM. Culturing with Trehalose Produces Viable Endothelial Cells after Cryopreservation. Cryobiology 2012; 64(3) 240-4.

[28] Brockbank KGM, Spyropoulos DD, Baatz JE, Chen Z, Greene ED, Campbell LH. Storage and Distribution Methods for Products Based upon Engineered Cell and Tissue Models. Abstract, 18th Annual Hilton Head Workshop & Annual Engineering Tissues Workshop, Hilton Head Island, SC., 2014.

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