

Eukaryotic Cell Storage and Retrieval at -80C: The Evidence for and Against Long Term Survival Rates

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Ideal environmental conditions for the storage of biological specimens are discussed in this brief article. Proper conditions for sample storage must take in account the methods for storage, documentation, retrieval, as well as the temperature and time that the specimens are intended to be stored. Most specimens are stored in a secure area called a biorepository that is meticulously organized and documented to enable efficient retrieval of any specimen. The storage conditions in the ideal biorepository will prevent or drastically minimize specimen degradation. The ideal biorepository will be an efficient, computer controlled, user-friendly storage system such as the BIOPHILE, Inc. (Charlottesville, VA, <http://biophileinc.com>).

Storage of Living Cells

Cryogenic storage of cells is a major activity in most research laboratories. Cryogenic temperatures are defined by the National Institute for Standards and Technology as temperatures lower than -150° C (-238° F or 123° above absolute zero on the Kelvin scale). In general, living cells will show less degradation in quality when stored in very cold, or cryogenic, temperatures. For example, human haematopoietic stem cells retain engraftment potential after extended (5–14 years) after cryostorage, but some loss of viability can be observed (1). However, liquid nitrogen freezers are inconvenient to use when they are compared to -80°C storage in conventional freezers.

Scientific fact does not support the popular opinion that all cells require cryogenic temperatures for long-term viability. Despite the generally held notion that all cells have better long term survivability at -130 to -150°C, many cells have been shown to survive in the frozen state at -80°C for many months, even years. For example, lymphocytes frozen in polystyrene tubes in a cryoprotectant consisting of 5% DMSO and 62% plasma remained viable for over sixty days as assessed by response to phyto hemagglutinin (PHA) and pokeweed mitogen (PWM) (2). The American Red Cross has shown that red cells, cryoprotected with glycerol and stored at -80°C demonstrate excellent post-thaw recovery and *in vivo* survival (3). Rat bone cells stored at -80°C for four months showed good survivability as measured by responses to hormones (4).

The Effect of Temperature Fluctuations on Cell Viability

Cell survivability in the frozen state may depend not only on the durability of the cell, but possibly more importantly the constancy of the temperature over time. Temperatures above -80°C have generally been used for the storage of tissue sections, plasma, DNA, RNA, proteins, and other biological material. Temperatures from -80°C and below have been used to preserve living organisms that can survive cryopreservation and yet still

remain “alive” (5). The principal reason that cells might not have demonstrated good long-term survival at -80°C is that until the advent of the BIOPHILE, there was no method to assure constant cell temperatures throughout the storage life of the cell. Inadvertent increases in temperature can take place when cells are temporarily removed by individuals “browsing” in the freezer, or by temperature fluctuations brought about by leaving the door open to the freezer for several minutes (Figure 1).

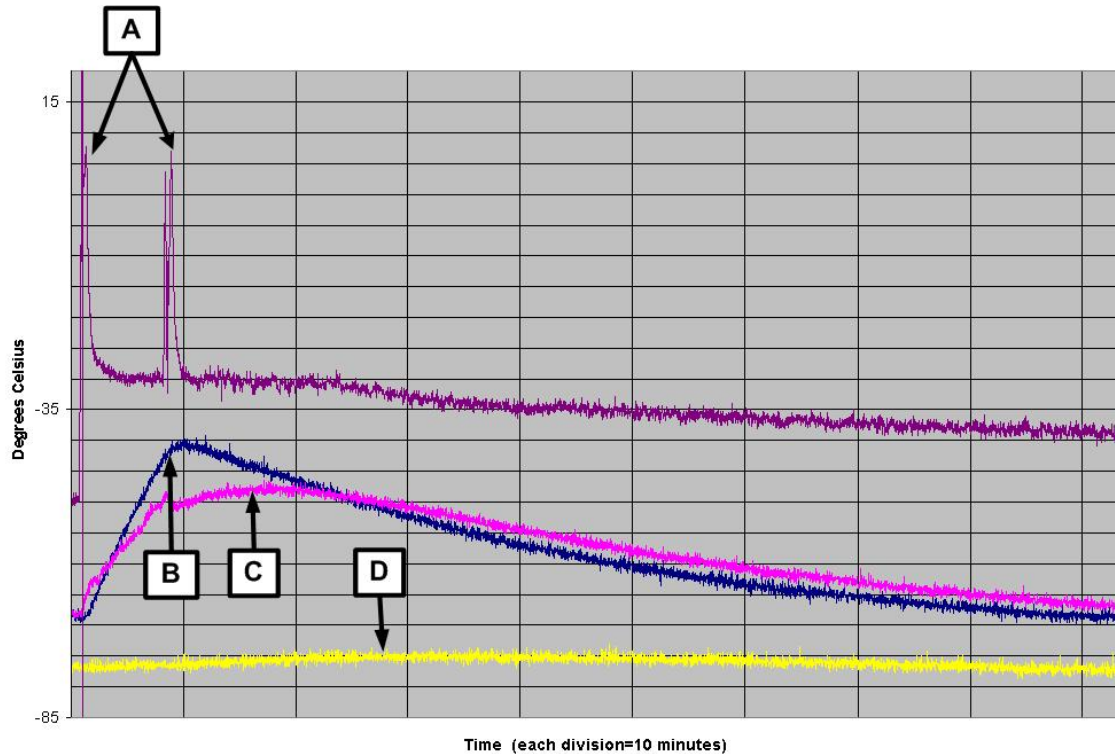


Figure 1: Temperatures inside a standard -80°C freezer were monitored by thermocouples over a several hour period. The door of the freezer was opened for three minutes on two occasions (marked with arrows on line A) causing a sudden increase in cabinet temperature. A deep well microplate containing thermocouples was removed for two minutes and then replaced in the freezer (lines B and C). The temperature in the well of the microplate required over two hours to return to a stable -80°C temperature. An identical thermocouple arrangement remaining in the freezer (line D) shows a 3°C thermal uptake as well. Crystallization may occur in the process of slow re-warming of specimens despite the fact that they are maintained at temperatures below freezing. This phenomenon (sometimes called "recrystallization") has can cause more damage than the initial cooling process. The many nuclei formed when cooling in the -80°C range can cause massive ice growth when rewarming in the -80°C to -40°C range. This is called "the devitrification problem." Thus, maintaining cells at a constant temperature at or below -80°C may be ideal for the long term storage of many living cells.

There are two major physical forces that disrupt the integrity of specimens that are frozen and then thawed for use. Ice crystals have been shown to denature proteins, disrupt cell membranes, rip apart cellular components, and cause concentration of solutes in the liquid phase around the forming ice crystals. The second harmful force in a freezing

specimen is the rapid change in osmotic forces that result from soluble substances as they migrate out of the forming ice crystal. High concentrations of salts that are expelled from the forming crystal lattice become toxic to cells. Cryopreservation is the science of protecting complex organic molecules and living systems from damage at ultra low temperatures by favoring conditions that prevent the formation of ice crystals. Rapid freezing of water in the presence of molecules that inhibit crystal growth results in vitrification, or the formation of a glass like state for water (water that does not organize into crystals). However, rapid freezing can also prevent osmotic equilibrium between the intracellular and extracellular environments. The term “vitrified water” has been loosely used to describe the state of ice that results from rapid freezing of a specimen that produces very small ice crystals. However, true vitrified water contains no ice. Vitrification has also been applied to organic substances like ethylene glycol or glycerol, which can favor vitrification, but can also become toxic to cells if present in high enough concentration.

Advantages of vitrification over freezing also include less osmotic shock from solutes that are excluded from the forming ice. High osmolar forces from concentrating solutes can deform proteins, disrupt biological structures, and even cause catabolic destruction of proteins. Vitrification is not the only method of minimizing ice damage. Ice damage can also sometimes be reduced by removing water, by reducing the freezing temperature, and by other means to limit the amount of ice and the size and location of crystals. The disadvantages of vitrification include the need for technology that promotes very fast cooling. In addition, in order to preserve the sample in the vitrified format, the temperature must be kept below the glass transition temperature for water. Furthermore, sample thawing must be rapid and in a controlled fashion to prevent ice formation, and hence additional damage as the specimen warms through the glass phase into the crystallizing phase for water.

Controlled Freezing and The Effect on the Storage of Living Cells

Cooling specimens in a slow and reproducible way ensures the maintenance of sample integrity and/or cell viability. For example, Shariatmadari et al demonstrated that a fluorescent signal from green fluorescent protein was lost in tissue that was rapidly frozen (isopentane immersion at -70°C) as compared to slowly frozen tissue (4°C for 24 hours and then placed in a box covered with cotton wool at -70°C) (6). Cooling rates of -1 to -3°C per minute will provide maximum viability for most eukaryotic cell cultures (mammalian cells, insect cells, and other higher organisms). Cells placed in vials that are stored in BIOPHILE trays that have been inserted into the BIOPHILE cool at a rate that approximates -1 to -3°C per minute.

Summary and Conclusion

The use of a freezer that provides constant temperatures over the entire storage period could allow living cells to survive for months and possibly years at -80°C . A constant temperature obviates the recrystallization that can occur in the temperature zone between -80°C and -40°C . An automated frozen storage system, such as the BIOPHILE, may not only provide ideal storage conditions for biorepository specimens, but also provide an

organizational infrastructure that will assure that viable high quality samples will be there when they are needed.

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