Cryopreservation of Biological Materials – The Viability Imperative.

Biological materials change and deteriorate with time, that is the nature of life. In order to preserve biological materials for later study or use, some means of halting these processes must be employed that will not fundamentally alter the nature of the material.

The most effective means of preserving biological materials is by freezing and subsequently storing at low temperature. Biological materials vary widely, therefore the only effective biological repository is one designed with reference to the mechanisms and bio-thermodynamics of the freezing process and an understanding of the issues associated with storage temperatures.

There is no such thing as too cold when cryopreservation is discussed. Once a substance enters solid phase, further cooling simply reduces the energy content of the material, or may cause structural changes. Critical temperatures for biologicals (the temperature below which no physical or chemical changes will occur) will typically be far below the fusion point – keeping the samples frozen is simply not sufficient.

From a good practice standpoint, the sample must be stored at a temperature sufficiently below any critical temperature such that normal operation of the storage system will ensure the samples are maintained below such temperature. In addition, the failure mode of the system employed must allow sufficient time for corrective action in the event of failure.

The fact that it is not a simplistic exercise to successfully freeze any living cell is what makes this enterprise so challenging. It is also key to the decision making which should take place every time any cell is frozen for subsequent future use.

Any time we look to preserve anything, the following questions should be asked, understood and answered in order to determine the level of care that needs to be taken in the preservation process;

- What is being preserved?
- How long is it necessary to keep it preserved?
- Why is it being preserved?
- Do we know all of the potential future answers to question 3 at this time?

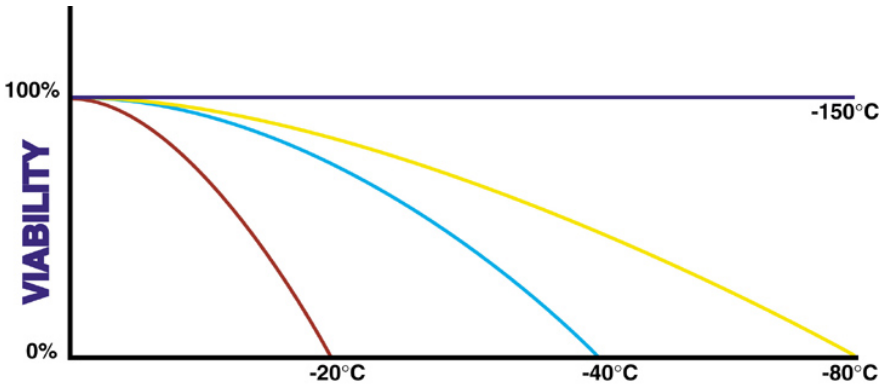
the last question being the most critical. Why? If we don't know now, all of the potential uses for the product that we are preserving, isn't it incumbent upon us to ensure that the maximum potential is preserved for future exploitation?

Consider this – 100 years ago, all preservation was fundamentally carried out as a reference source for future study. If an intrepid adventurer forayed into the jungle, returning with a strange animal specimen, comparison to the great museum reference collections of stuffed animals would indicate genus, type and whether the animal was previously known. This was the raison d'etre of the collections and as such they admirably fulfilled their purpose. But wouldn't a zoo be better? The current analogue is the storage of cells without either adequate care during the freezing down process or too high a temperature during subsequent long-term storage. Either will cause the cell to lose the potential for future life. DNA and RNA may well be preserved intact but only in a form suitable for future reference comparison – and hence we are back to the museum, not the zoo.

In order to protect the viability of the cells for the future, we should look at the mechanism by which cells freeze. Much of the difficulty involved in cryopreserving cells is derived from the anomalous properties of the cells' main constituent – water! We are all aware that ice is less dense than liquid water, mainly due to the competing effects of fusion energy and the hydrogen bonds present in the molecular make up. The expansion of the liquid inside the cell, if unchecked, will cause serious damage when the cell freezes. Another property (useful this time) is the fact that water, as it freezes, will exclude any solute from the solid phase. It is this property more than any other which enables us to manage the freezing process and retain cellular viability.

As the liquid containing the cells begins to freeze, the concentration of any solute present will begin to increase in the unfrozen fraction. As this happens, the osmotic potential across the membrane of the cell begins to increase and one of two things must occur. Either water must egress for the cell to reduce this potential, or solute must enter the cell. The rate at which this transfer occurs can be controlled by the external cooling rate during the freezing process. In general, the aim is to dehydrate the cell to the point at which the remaining liquid within it is so viscous that the molecules have insufficient energy to order themselves into any long range (or crystalline) structure, and instead form an amorphous solid (or glass). This "phase" change (physicists and thermodynamicists still argue about whether this glass formation is a true phase change) occurs at a point known as the "Glass Transition" temperature (or Tg) and it is important because below this temperature no diffusion can take place within the cell and its surroundings. Without this diffusion, the biological clock stops. It is fair to say that above this temperature all storage achieves is to delay the process of degradation. Cellular death is merely postponed, not avoided. Below this temperature the integrity of the sample can be maintained, at least theoretically, for eternity.

Graphically this can be represented like this



The published temperature for the glass transition point varies slightly from researcher to researcher but is generally regarded as being -132°C.

Given the facts that: a) only if the temperature of the sample remains below -132°C is the long term viability of the sample maintained and b) transitions in either direction through this phase point will cause loss of viability, it becomes imperative that the sample be held well below this critical temperature for the whole storage period. In fact most researchers will quote -150°C as the critical temperature for most cell products to allow sufficient safety margin for normal working operation of the storage freezer. Storage in liquid nitrogen at -196°C is an obvious answer. This method allows for a 60 plus degree safety zone. Liquid storage, however, has some disadvantages, personal safety when handling liquid full racks and potential sample container rupture if liquid enters for example. The most important consideration is sample to sample pathogen transfer. There have been several documented cases of cross contamination in liquid storage, and although quarantine is an option, this only accounts for pathogens we know to look for now. 20 years ago (and that is not long when we speak of eternity!) few if any looked for HIV, Hep C, Prions etc..
Storage in the vapor phase has often been looked at as a solution to these issues. In the past, problems associated with vapor phase temperature and gradient, safety periods in the event of LN2 outage and liquid usage rates have all been raised as arguments against this method.

The Eterne storage freezer from Chart MVE BioMedical is a liquid nitrogen cooled freezer operating in the vapor phase at or near liquid nitrogen temperatures.

Stored samples are placed in racks sitting on a rotating platform above a reservoir of liquid nitrogen. The design of the tank ensures that a very high proportion of the surface area of the freezer is protected by a vacuum space. This vacuum protection is the reason very little energy (heat) enters the tank from the

environment, meaning the liquid nitrogen in the base of the tank evaporates at a very slow rate. All of the energy entering the tank is conducted by the rotating tray and the sample racks to the liquid reservoir where it is absorbed. The efficient thermal management of this stray energy, plus the very limited quantity of energy allowed in to the system ensures that the temperature within the tank is extremely low – in the order of -190°C, more than 55 degrees below the critical point for safe biological long term preservation. This temperature is maintained throughout the storage compartment with very little gradient and the advanced thermal design reduces nitrogen usage to less than half that required for a standard tank in vapor mode. In addition to yielding very low LN2 usage rates, this also ensures that temperatures are maintained even with the lid removed for up to two hours and, when filled, the unit will maintain temperature for up to a month with no additional filling.

For more information on the Eterne freezers, or the general topic in this article please contact Lois Tuma, MVE BioMedical Customer Service, 952 882 5230.