Precision in Cryopreservation – Equipment and Control

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1. Introduction

1.1 Different samples may have differing cryopreservation requirements

For any cryopreservation protocol there are five key questions that govern the methodology and logistics of the freezing and storing process.

- What is to be stored?
- How many batches are to be stored?
- What is the expected duration of storage?
- What properties are the retrieved samples required to possess?
- Are there packaging requirements in addition to those dictated by the cryopreservation process?

Reduction of temperature results in the retardation of metabolic processes and this can, in some circumstances, provide sufficient stability for the required period of storage. However, at temperatures below 0 °C the biological effects of cooling are dominated by the crystallization of ice: typically, water constitutes around 80 % of tissue mass. Freezing is the conversion of liquid water to crystalline ice but the term is commonly misused in circumstances where samples are cooled below their expected freezing point but without the formation of ice, for example by supercooling or by vitrification. The result of the freezing of water in a complex solution is that the concentration of the solutes in the remaining liquid phase increases and some solutes may precipitate if their concentration exceeds their solubility limits. This realisation provides two potential mechanisms of damage: direct mechanical effects of the formation of ice, and the rise in concentration of dissolved solutes.

In 1948 a method was discovered that permitted the freezing of many types of animal cells with good post-thaw recovery of living cells: Polge, Smith, and Parkes (1948) showed in a landmark paper that adding 10-20 % of glycerol enabled avian spermatozoa to survive freezing at -80 °C. Theories of freezing injury that were current at the time envisaged ice crystals damaging the cells and intracellular structures, and because glycerol increased the total solute concentration in the system, the amount of ice that formed was reduced. A little later, in the 1950s, Lovelock (1952) showed that the increase in concentration of salts as the volume of the suspending solution decreased was in fact the dominant damaging mechanism: salt concentration, rather than ice formation, was a major cause of freezing

injury to cells. Subsequently other cryoprotectant solutes were explored along with different rates of cooling, resulting in solidification of the stored samples but with a range of mixtures of ice and vitrified solid in the stored samples.

The physical nature of the sample dictates the thermal transfer characteristics of the cooling process for that specific sample and either the physical size or cell-type will affect the appropriate cooling rate and other parameters of the cryopreservation protocol. Similarly, the physical type and ultimate intended use of the sample (for example dose requirement in the case of future therapeutic use) will determine the size of the individual packaging. An additional layer of packaging may be necessary to prevent microbiological contamination – so-called 'double bagging'. Likewise the ultimate destination of the sample will also dictate the care required during the freezing process and the conditions necessary for long-term storage. Some tissues and most larger biological samples are currently difficult or impossible to cryopreserve successfully and new techniques, such as Liquidus Tracking (discussed later in this chapter) may address some of the problems associated with cryopreservation of these types of sample.

It is sometimes the case that the ultimate use of the samples stored is not known at the time of the initial collection and storage and sometimes the significance of particular samples may change with time. However, in many cases, the potential of the stored samples is fixed or limited at the time of selecting the cryopreservation and storage methods. The importance of these choices will be covered later; however it is pertinent to note here that the storage process may have an important impact on the value of samples when they are recovered from storage; changes in the properties of the recovered samples may be irreversible and this is therefore a key to maximising the sample's potential.

The term "viability" is frequently used in the context of cell and tissue banking. Strictly speaking it means the potential to exhibit the signs of life at some future stage, whereas it is often misused to mean the extent to which a sample demonstrates attributes of life at the present time. But that is "vitality" not "viability." However, it is also the case that not all the attributes of life are exhibited by all living things, and the possession of one attribute does not imply the presence of them all. In fact, few of the properties that characterise "life" can be measured quantitatively. The term is best avoided; functional measurements should be named to describe what they actually measure: membrane integrity; a specified metabolic function; ability to reproduce. In addition there are obvious cases where the tissue does not have to be alive in order to function; in bone for example. But equally, in many cases fully functional survival is paramount; the haemopoietic stem cells in cord blood will not graft in the recipient if the cell concentration is lower than a threshold value. In such cases a low total recovery of living cells in the thawed sample will limit the use of the thawed sample. Another common situation is where samples are stored in order to ensure that a supply of identical cells will be available throughout a long-term study. Although it is possible to regrow new cell batches from recovered samples, repeating this process can lead to progressive degradation due to mutations.

1.2 The physics of freezing

The process of freezing is ultimately simple; it is merely the application of an environment that removes energy from the sample over a period of time and changes the physical state of

water in the sample from liquid to crystalline. Crystalline water (ice) excludes the solutes previously dissolved in the water, resulting in two potentially dangerous mechanisms – direct effects of ice and secondary effects in the solute composition. At a sufficiently low temperature all biological activity is prevented and the physical state of the sample is preserved. In simple cases, where the only requirement is to preserve the physical state or where cellular structure is absent (viruses, DNA etc.), that is the end of the story; physical deterioration can be prevented at relatively high temperatures, and in many institutions worldwide this task is completed in the banks of -80 °C refrigeration units that proliferate in medical and biological research establishments.

The preservation of living cells and tissues and the post-thaw ability of cells to proliferate and thrive are determined by a number of factors: the laboratory techniques and the thermodynamic processes that a sample experiences during processing and freezing; the environment in which it resides between freezing and the ultimate use post-thaw. The potential of many samples is severely limited at this stage by the choices made by, or enforced upon, the technician regarding the freezing protocol. It may be that some stored samples lose significant value due simply to the omission of a few simple additional steps.

The cryopreservation process has two main aims. The first is to reduce the temperature of the sample to a point where biological stability is achieved. The application of an external cryogenic environment will remove energy from the sample and create a very low-energy solid state within which biological and chemical activity are limited or prevented altogether. The second is that during the freezing process it is necessary to prevent the formation of intracellular ice crystals: such crystals damage the cellular structure and can lead to limited post-thaw recovery and post-thaw failure of the cell sample to function as required. Additionally, the protocol must take into account the stresses to which the cells are exposed during the freezing process (dehydration, hypothermia, chemical toxicity, and solute concentration) and the potential for an apoptotic response post-thaw.

The objective, therefore, is to create an environment in which, as the sample is cooled, the chemical composition inside the cell, is managed in such a way as to create an intracellular composition with a lower freezing point than the applied environment, whilst maintaining an external suspending composition that is able to solidify at the same temperature. The balance between the internal and external environment is managed chemically via the solutes in the micro-environment and thermodynamically via the application of an energy reducing (cooling) macro-environment. It is the combined action of these two factors that determines the success or otherwise of a cryopreservation protocol for the conservation of vitality.

The appropriate solute composition is created by including cryoprotective agents (CPAs) in the medium. These operate in one of two ways: either they modify the extracellular composition or alternatively they also replace some of the intracellular water. The first mechanism involves the addition of non-penetrating CPAs such as trehalose, polyethylene glycol (PEG) or Polyvinyl-pyrrolidone (PVP), to the medium. The second mechanism requires the addition of penetrating solutes that can traverse the cell membrane, such as glycerol, ethylene glycol and dimethyl sulphoxide (DMSO). Since water does not retain solutes when it freezes, a solution at equilibrium with ice will vary in osmotic potential as it freezes and because of this, the micro-environment of a cell will require either the cell to lose water to the environment or exchange water for CPA molecules, thereby maintaining

osmotic balance. The concentration of intracellular material lowers the effective freezing point of intracellular material and, provided the external temperature is correctly managed, prevents the formation of intracellular ice. As such, the creation of ice crystals within the cell is avoided. At temperatures below -130 °C (close to the glass transition temperature of the medium) the residual liquid has too little energy to orientate into long range molecular matrices and will form short range semi-solid structures; i.e. an amorphous solid or glass. At this point there is no possibility for significant chemical transport; biological activity, and hence deterioration, effectively ceases.

The options for control of this process are the chosen CPA and its concentration, and the cooling rate. Water and solute permeability are temperature dependent and nominally the higher the concentration of extracellular CPA, the less ice will form during cooling. With a very high applied concentration of CPA, very rapid cooling without the formation of ice may be possible – a process that is known as vitrification. At the other extreme, lower CPA concentrations that allow ice to form, require more precisely managed cooling rates which can be provided by programmable controlled rate freezers. The issue here is the toxicity of the applied CPA since high concentrations, even for short periods, can lead to excessive dehydration and high cell stress, whereas lower concentrations may involve prolonged cellular exposure to essentially toxic material. DMSO, for example, is an organic solvent and has been linked to cellular mutation. The choices made for the preparation and subsequent freezing of cells is a complex balance between thermodynamic and biochemical variables, the choice and management of which can have a profound effect on the post-thaw recovery of living cells and hence the value of the sample.

1.3 Long term cell survival and contamination

All biological materials will, without intervention, naturally deteriorate, and if they are to be preserved it is necessary to utilize a method that will preserve both morphology and functionality while preventing any alteration of the fundamental nature of the material. The most common methodology available for this is cryopreservation. Biological materials, however, have widely different properties and in order to create a truly effective cryopreservation protocol, it is necessary to consider these properties as they affect the preservation of vital characteristics both during the freezing process and the subsequent environment in which the samples are to be stored long term.

Regarding the minimum storage temperature, no temperature is too cold. Once a sample is frozen and the residual liquid phase has vitrified, further cooling simply reduces molecular energy and vibration. It is possible for short-range structural changes to occur at a molecular level, but they do not affect post-thaw biological properties. It is worth noting that because the cell micro-environment within a frozen sample is chemically different from the majority of the frozen material, biological activity may continue, albeit slowly, at temperatures several degrees below the freezing point of the material.

The minimum melting point of the multi-dimensional phase diagram for typical cryopreservation media occurs at around -80 °C but the cell contents do not finally solidify to an amorphous state until around -120 °C. It is not sufficient simply to keep the samples frozen because, at a micro-environmental level, if the material retains the ability to diffuse it may also degrade, albeit at a much reduced rate. The glass transition temperature is therefore regarded as the "critical" temperature if truly long term storage is required

Best practice dictates that freezers should maintain sample temperatures as far as possible below this critical temperature. By storing well below the critical temperature, transitory warming events above that temperature can be avoided during sample handling, retrieval, storage and in the event of any disruption to the availability of cryogen or power. Freezing a sample in such a way as to maintain maximum biological potential is not a trivial task, and the same care applied to this process should be brought to bear when designing and building storage environments.

The key considerations when looking at a cryopreservation process were listed at the beginning of this chapter. Clearly, the process should be able to maximise the potential for use after processing and storage. Because the future use may be unclear, the preservation and storage procedures should be designed to provide the best possible opportunity for future exploitation. The storage of cells without either adequate care during the initial cryopreservation process or at too high a temperature during subsequent long-term storage are key problems that should be avoided and when the purpose of storage is to maintain biological potential, it is vital that the mechanism of freezing injury be considered.

As the liquid in which the cells are suspended begins to freeze, any solutes in the unfrozen solution become more concentrated and this results in a depression of the freezing point of the remaining solution. The result of this, when the temperature is reduced, is that the cells are exposed to a solution of progressively higher concentration. The increasing concentration increases the osmotic gradient across the cell membrane which results in water leaving the cell in order to maintain balance. Hence, controlling the cooling rate provides a mechanism for controlled dehydration of the cells. Eventually the aqueous phase is so viscous that there is insufficient energy available for the water molecules to form a crystalline solid and the solution becomes an amorphous solid or glass. The temperature at which this condition is reached is known as the "glass transition" temperature (Tg). Once the sample is below this temperature, diffusion within and without the cell stops and the sample is biologically inert. At temperatures below Tg the sample can be maintained indefinitely; other physical interactions, such as background radiation, may have an impact on extremely long-term storage but such effects are probably without any significance in practice (Glenister et. al., 1984).

Unlike freezing, the glass transition is not based upon a thermodynamically defined phase change but rather on the observed dramatic change in viscosity that occurs in cryoprotectant solutions typically at around -120 °C. It is important to ensure that samples are maintained below this temperature throughout the storage term. A temperature of -150 °C is typically stated to be the critical storage temperature for cell products since this temperature provides a reasonable safety margin to ensure that that samples remain below the glass transition temperature during transitory events such as handling, but in practice, storage in liquid nitrogen at -196 °C is a convenient and reliable way to meet this requirement; moreover the additional safety margin provides even greater sample security.

However, storage in liquid nitrogen is not without its disadvantages which include the risk of explosion during warming should liquid nitrogen have entered the vials. Microbiological cross-contamination is another hazard of storage in liquid nitrogen (Byers, 1999) and may lead to the application of a secondary enclosure ('double bagging'). Storage in the gas phase has been advocated to avoid these problems. In the past, the temperature gradient in the vapour phase of liquid nitrogen refrigerators has been a problem, and there may have been

increased vulnerability to inadequate amounts of liquid nitrogen between refills. Modern high-efficiency liquid nitrogen cooled vessels now allow storage in the vapour phase without these problems. These vessels are vacuum insulated and the surface area that is not insulated by the vacuum is minimised ensuring that the evaporation rate of liquid nitrogen is kept low. Restricting the amount of energy entering the vessel ensures that the temperature in the vapour phase is maintained close to the liquid nitrogen temperature. The upper region of the refrigerator, close to its access point where the temperature would otherwise be higher, can be efficiently cooled if it is ensured that the heat exchange surface extends right from the bottom to the top of the refrigerator: gas phase temperatures of around -190 °C can be achieved.

The weak point in the process of maintaining safe low temperatures for samples focuses on the time in transport to and from its storage. Small samples of low thermal mass, such as vitrified straws, can warm at the rate of thousands of degrees Celsius per minute and regulatory inspections requiring the removal of samples for identification can be another weak point.

1.4 Traceability

Under most regulatory environments, a rigorous sample tracking system is a key and mandatory component of compliance. It is vital that the individual location of any sample is recorded accurately, and that the sample is labelled with a unique identifier such that the identity of a sample at any location can be verified. For many research and therapy provision operations it is also necessary to have all processing, analytical and, if relevant, patient data linked in a central database.

Labelling can be a challenge as sample containers can be small and the cryogenic environment hostile; however commercially available cryogenic-proof labels and label printing systems are readily available. RFID tags are also a promising solution.

Sample location databases should be organized hierarchically, such that the location of any individual sample can be readily identified; for example: Room / Freezer / Shelf; or Segment / Rack / Position of Box in Rack / position of Vial in Box; or Room / Freezer / Canister / Cane / Goblet / Straw position in Goblet.

Most regulatory environments require the label to include both machine and human readable identifiers (bar code plus text) and where a sample is stored in a secondary container (such as a blood bag in a cassette) it is vital that both the external container and the primary sample container be correctly labelled; see for example the European Directives 2004/23/EC and 2006/86/EC

Concomitant with good identification procedures are good location and retrieval methods and there are a number of commercial software systems available with varying degrees of sophistication to accommodate larger or smaller numbers of stored samples. However an often overlooked part of the storage process is the logging, monitoring and associated alarms. Recording the parameters of storage is sometimes seen only as a regulatory obligation but liquid nitrogen levels or temperatures and the performance of mechanical freezers is of front-line importance. Alarms that work in practice rather than in theory are vital additions to a comprehensive storage environment.

2. Types of technique

There are various options to consider when choosing the methodology and equipment for the cooling process. In conventional cryopreservation, where the intention is to control the rate of formation of ice in the material, it is necessary either to vary the rate of application of a cryogen when working against a constant warm environment, or to provide insulation or energy while maintaining a constant external cold environment. If insulation is used, the cooling rate at any point is approximately proportional to the difference in temperature between the sample and the environment as modified by the insulation and the change in specific heat of the sample as cooling proceeds. Thus, during the process, the cooling rate asymptotically approaches zero as thermal equilibrium is achieved. Applying variable energy to a sample in a cold environment allows the rate of cooling to be modified during the process. The aim is to maintain a composition within the cells that varies as cooling proceeds such that its freezing point remains below the applied environmental temperature. Alternatively, if the concentration of cryoprotectant is high enough, it may be possible to cool the sample sufficiently rapidly that ice cannot form – an approach called vitrification. The required cooling rate will depend on the cryoprotectant and its concentration, the latter being dependant on the concentration that the cells will tolerate. In general, very rapid but uncontrolled cooling is used. The new technique of liquidus tracking allows slow cooling and vitrification.

2.1 Freezing in mechanical freezers

Passive cooling uses insulation to moderate the cooling rate of samples that have been equilibrated with low concentrations of cryoprotectant and then placed inside traditional electromechanical refrigerators at -20 °C, -40 °C, -80 °C or even at lower temperatures. The cells are dessicated slowly during the cooling process. This method can be used for most robust cells but even under the best circumstances the post-thaw recovery rates may not be ideal. In addition, in most cases no instrumentation monitoring or recording of the process is provided. The variation of temperature within mechanical refrigerators is well known with one study reporting values of -43.5 °C to -90 °C in upright freezers (Su et. al., 1996). Since there is no active control during the process, it follows that the poor repeatability of the process can affect the cooling rate and hence the efficiency of the whole procedure. Variability might be improved if the local environment were more stable and protected from instantaneous variation due to external factors such as door openings etc. It is generally preferable to use a liquid nitrogen gas phase freezer for this approach since the internal temperature variation is small and the environment disturbed less frequently.

This approach to cooling and freezing is increasingly being used for material provision in pharmaceutical drug screening programmes as they move from supply by continuous culture towards a "cells-as-reagents" concept. In this approach, the cells are insulated in polystyrene containers as they are cooled initially to -80 °C and then transferred for cryogenic storage into liquid or vapour phase nitrogen. The need for rapid use of the cells for drug assays following cryopreservation, places an increased importance on the post-thaw quality of the cells. In recent work carried out at LGC (Teddington,UK) this has been shown to be compromised by this type of freezing where the cooling rates are not actively controlled but rely on the passive characteristics of the system. In particular, temperature fluctuations within the polystyrene container and the storage time at -80 °C can significantly impact the post-thaw recovery of the cells and their biological function.

2.2 Controlled freezing, protocols and seeding

Liquid nitrogen may be applied via a pressurised supply and cryogenic valve to create a very accurate cooling profile of temperature over time. This methodology offers the most options for optimization since the cooling rate can be varied at multiple stages in the process. As freezing proceeds the concentration of solutes in the medium increases causing cell dehydration in the sample.

As described in the opening section, cooling protocols are designed to manage the intracellular solute concentration. The key point is the nucleation temperature of the suspending medium - that is, the temperature at which ice starts to form. The ice is extracellular, resulting in an increase in the extracellular solute concentration and and hence an osmotic pressure difference between the intracellular and the extracellular solutions that leads to the withdrawal of water from the cells. It is important to recognize that under normal circumstances, solutions do not freeze at their freezing point; they freeze at their nucleation temperature, which is variable and depends on the availability of nucleation centres in the sample. The nucleation temperature is normally several degrees below the nominal freezing point.

Once the extracellular fluid begins to freeze, two major events occur. First, as explained above, the concentration of CPA increases in the fraction of the extracellular fluid that has not at this point frozen, and this causes the cells to dehydrate. Secondly, the temperature of the suspension where freezing has commenced rises towards the nominal freezing temperature and remains at or close to this temperature until the freezing process is complete. This is followed by a drop in temperature as the sample catches up with the temperature of the surrounding medium, but if the cooling rate is too rapid the intracellular CPA concentration may be insufficient to prevent intracellular freezing – with severe consequences for the cells

In order to avoid this hazard, the control program may be designed to allow equilibration of the sample and its suspending medium at a temperature marginally below the calculated freezing point and at this temperature the sample forced to begin to freeze by applying either a physical nucleation point via a cold instrument placed on the external wall of the sample container, or via a sudden, short-lived introduction of cryogen into the environment. This causes the sample to commence freezing. As the sample was originally held only marginally below the nominal fusion temperature, the cell experiences a much more moderate reduction in temperature when the fusion is complete and the temperatures re-equilibrate. After this, the cooling processes is started and continues with a temperature program that is designed to effect the necessary concentration changes to maintain the intracellular composition in the liquid region of the phase diagram. This process is called "seeding".

2.3 Vitrification

The process of vitrification usually uses the highest concentration of cryoprotectant that the cells in the tissue will tolerate and follows this by very rapid but uncontrolled cooling, usually by plunging the sample into liquid nitrogen. The crucial element is exposure of the cells or tissue to potentially toxic levels of CPA: too low a level or too short a time and ice will form killing the cells. If the levels are too high or the process time is too long, the chemicals employed will prove toxic to the cell and post-thaw viability will be limited. Since this process depends on rapid cooling, vitrification has only ever proven applicable for

samples with very small volumes, ideally those with very high surface area-to-volume ratios; for example cryogenic straws can fit this description. It is important to be aware of the Leidenfrost effect where a sheath of vapour will surround a warm sample when plunged into liquid cryogen, essentially insulating the sample for a short period of time. For many vitrification protocols however, even this short additional time period before the sample is vitrified has proven fatal to the cells due to increased toxic exposure to the CPA and decreased cooling rates. In conventional vitrification, very high cooling rates are achieved by exposing small samples directly to the liquid nitrogen. The sample is surrounded with as little physical material as possible to achieve the maximum cooling rates. With large samples, however, such high cooling rates are impracticable.

Although vitrification is normally associated with cooling rates in the tens of thousands of degrees Celsius per minute, slower techniques have been reported such as the S3 vitrification technique for blastocysts (Stachecki & Chen, 2008); this uses rates <200 °C/minute. But in fact vitrification does not necessarily require rapid cooling at all. It all depends on the dependence of the critical cooling rate required to prevent freezing on the concentration of the cryoprotectant. (Sutton, 1991). As the following section describes. vitrification can be produced at really low cooling rates.

2.4 Warming and thawing

It is usual to thaw cryopreserved or vitrified samples rapidly – typically by plunging them in a 37 °C water bath. The warming rate does have an effect on the recovery of living cells but this is not as great an influence as cooling rate is during cooling. In fact, optimum cooling rates have usually been determined using rapid warming so it is hardly surprising that rapid warming then gives the highest recovery! However, there are circumstances when the warming rate is of importance in its own right. The first is when the sample has been vitrified but is nucleated without a significant amount of ice being present. This is an unstable situation and in such circumstances the warming must be rapid to avoid intracellular freezing during warming. This consideration argues for rapid warming. The other situation occurs when the frozen material contains a significant amount of vitrified material, as is always the case in conventional cryopreservation. Glasses are brittle and the hazard here is that rapid warming will generate thermal stresses and cause the vitreous material to fracture. This will not matter greatly with cell suspensions where a fracture running through the sample is unlikely to traverse many cells but it is very important when the extracellular matrix must be intact – as it must, for example, in grafted blood vessels and heart valves. The solution here is to warm through the vitreous zone, that is from -196 °C to -123 °C, relatively slowly: once above the Tg there is no hazard from fractures and the sample can be warmed as rapidly as you like. A convenient way to do this is to allow the sample to warm slowly in a -80 °C refrigerator or packed in solid CO_2 until its temperature is at above -100 °C. Alternatively the sample can be surrounded by a layer of insulation during the initial stage of warming in room air. A warming rate of around 50 °C/minute up to -100 °C was 'slow' enough to prevent fractures in cryopreserved rabbit carotid arteries (Pegg et al., 1997).

2.5 Liquidus tracking – A new method

The controlled-rate freezing process achieves its results by preventing the formation of intracellular ice. In some samples, however, even extracellular ice can be severely damaging.

An example of this is articular cartilage. Isolated chondrocytes can be cryopreserved using conventional techniques (Pegg et. al., 2006a) but results when attempting to cryopreserve chondrocytes in situ have proven to be very disappointing. It was found that traditional cryopreservation results in the formation of ice crystals within the chondrons and not just in the acellular matrix (Pegg et. al., 2006b) which might have been expected from experience with conventional cryopreservation. In articular cartilage it is important to prevent both intracellular and extracellular ice. With this requirement in mind, the most appropriate cryopreservation approach would appear to be vitrification; that is the prevention of any ice formation at all. However, it will be clear that conventional vitrification is out of the question because of the heat transfer problems with bulky samples. Liquidus tracking (LT) provides a new approach to this problem.

During conventional cryopreservation, with a moderate concentration of CPA (say 10 %w/w) and relatively slow cooling (say 1 °C/minute), the cells are exposed to gradually increasing concentrations of cryoprotectant as progressively more extracellular ice is formed. The instantaneous CPA concentration is determined by the temperature according to the phase diagram of that specific system. The idea of LT is to control the instantaneous concentration of CPA throughout the cooling process so that the CPA concentration follows the liquidus line by external control rather than by progressive freezing of the medium. In this way the medium remains just above its freezing point at all times and no ice is formed. It is important to note that the cells are exposed only to the concentrations of CPA that they would experience during conventional cryopreservation. And we know that isolated chondrocytes in suspension can be cryopreserved by standard methods. In effect, the LT process takes advantage of the decrease in cytotoxicity of cryoprotectants as the temperature is decreased: hence, rather than starting with a very high concentration of cryoprotectant, the LT approach controls the concentration dynamically throughout the cooling process. In this way, vitrification can be achieved without using the extremely high concentrations of cryprotectant at the start of the process and without the need for rapid cooling. Of course, allowance has to be made for the time that diffusion of CPA into the tissue takes and this can be very considerable. On the other hand, if an organ can be perfused with the cryoprotectant solution, via the vascular system during cooling, then the diffusion distances will be very short and mass transport delays much less significant. In practice, when designing an LT process for a particular tissue, it is crucial to determine the concentration that is actually achieved in the tissue as the process continues and to adjust the concentration/ temperature/ time program to achieve the desired tissue concentration at all stages of the process. This necessitates slow cooling, commonly of the order of 0.1 to 0.3 °C per minute. The cooling of the samples can be achieved in a conventional controlled rate cooler and the solution composition can be controlled by standard peristaltic pumps, the whole system being under computer control – see section 3.7 below for a discussion of the methods that are now available for research use.

3. Types of equipment

Due to the many different types of samples that can be cryopreserved and their differing sensitivities, a number of different techniques and types of equipment are used.

3.1 Minus 80 °C and lower: Mechanical freezers

Mechanical refrigeration always applies the same methods no matter the degree of cooling desired: a gas is passed through a compression system and liquefied. The energy which is

released during this liquefaction is dissipated to the environment via heat exchanger coils. The liquid is then passed through cooling coils within the freezer chamber and absorbs energy from the chamber as it vaporizes. It is the vaporization process that creates the cooling effect. As lower temperatures are required, lower liquid point gases must be employed. In order to liquefy these gases, higher pressures are required and often the liquefaction cannot be completed in a single process; this results in larger, multiple compressors being employed.

The most commonly used freezers for cryogenic purposes are upright, front-opening freezers with a cold point at a nominal -80 °C. It should be noted that there is no biological significance for this temperature, merely a physical significance since it approximates to the sublimation temperature of dry ice (solid carbon dioxide, -79 °C). This type of freezer can be employed to store biomaterial in which living cells are not a prime concern, or when it is to be stored for only a short period of time. This type of equipment is intended to be for transactional storage – holding material required daily and which will either be consumed or transferred to more appropriate conditions within a short time - 6 to 12 weeks typically.

The front opening design, while adding considerable convenience, creates a significant issue with temperature stability and variability. Because cold air is significantly heavier than warm air, opening the door causes massive air exchanges and temperature rises in the sample area in a short period of time. In addition, because the compressor systems run on a very high cycle time, there is little spare capacity to effect a cooling after the temperature has risen and it can take some time to return to equilibrium after a warming event. This property is similarly exhibited when the freezer is in normal operation and as has been previously noted, there can be significant temperature variations. The use of deep drawers within the refrigerator for the storage of samples is helpful in reducing the loss of cold air when the door is opened.

Because of the high cycle times, compressor failures are quite common and expensive to repair. It should also be noted that as the energy removed from the sample area is 100 % dissipated into the room in which the freezer is located, the term cost of operating a unit such as this should take into account not only the electricity consumption required for the compressor system, but also the significant air conditioning costs associated with the expelled heat from the freezers. If this energy is not removed by air-conditioning, the freezers become less efficient as room temperature rises, compressors are required to cycle even longer, power usage rises and compressors fail more quickly. Environmental management at a macro as well as micro level is therefore important.

3.2 Alcohol bath freezers

These commonly used laboratory units are essentially refrigerated circulators. A reservoir of cooling medium (normally an alcohol) is passed through a cooling system and re-enters a reservoir, reducing the temperature. The degree of refrigeration applied and the flow rate through the cooling coils determine the derived temperature of the reservoir. The relatively large volume of cooling liquid creates two noticeable effects: temperatures are very stable due to the large heat capacity of the available fluid and cooling rates can be controlled very accurately for a similar reason. The corollary to this however is that the rates achievable are very low and so rapid (> 1 °C/minute) rates are very hard to achieve. In addition, alcohol bath freezers are normally limited to temperatures above -80 °C.

3.3 Liquid nitrogen vessels: Liquid and vapour

Storage of important biomaterial in liquid nitrogen at -196 °C is widely practised. This method allows for a 70 °C plus safety zone when considering the -120 °C threshold for long-term storage; the significance of -120 °C, the glass transition temperature, has been previously discussed. Liquid nitrogen storage does provide the greatest safety zone. However, it also presents a number of problems, including personal safety and potential microbiological cross-contamination via the liquid nitrogen.

Storage in the vapour stage is felt to address these issues but it does come with its own set of problems. The vapour is not as cold as the liquid nitrogen itself and as such the 70 °C safety margin is diminished. However, modern vapour storage vessels use carefully designed vacuum insulation to minimise the heat leakage from the environment into the vessel. This allows the vessel to maintain a vapour temperature at around -190 °C resulting in samples still being maintained at a safe distance from the glass transition temperature. Efficient designs also result in very low liquid nitrogen usage and temperatures can be maintained for up to a month without additional filling; temperatures are even maintained with the lid removed for short periods.

3.4 Controlled rate liquid nitrogen freezers

As described previously, up to present times, the controlled rate freezer offers the widest control options for a freezing protocol. With a truly variable application system for cryogen, most sample sizes can be easily accommodated and rates from the very slow (< 0.1 °C/minute) to in excess of 50 °C/minute are both achievable and controllable. Sample size, container dimension, cell volume, membrane permeability etc. are all variable factors. As the controlled-rate freezer allows complex, fully controlled temperature versus time profiles to be created, protocols can be designed that are appropriate to the cell type and cryoprotectant concentration. Additional steps such as pauses for manual 'seeding' or rapid plunges to initiated freezing can be added to the profile. Transition to different rates can be triggered from the chamber temperature or representative sample; triggering the transition from the sample temperature can help remove variability introduced by different sample loads..

From an instrumentation standpoint, the programming and record-keeping intrinsic within the system meet most external compliance standards and optional software packages are generally available to enhance this aspect beyond the current requirements of any legislative authority. The fact that it is possible to optimise processes for every unique cell type together with the compliance aspect lend great versatility to this type of instrument in most application areas.

3.5 Equipment for conventional (high cooling rate) vitrification

Such protocols call for extremely rapid solidification of the sample, typically by plunging it directly, and in a somewhat uncontrolled manner, into liquid nitrogen. Intracellular ice formation is avoided by the application of very high concentrations of CPA. Equipment such as the VitMaster (IMT ltd.) can be used to increase the cooling rate. This uses negative pressure to depress the freezing point of liquid nitrogen to below -205 °C thereby increasing the cooling rate. Several open techniques have been developed to minimise the sample volume and achieve high cooling rates; for example the Cryotop method which uses a thin

film strip to hold the sample. These open systems typically expose the sample directly to the liquid nitrogen which assists in achieving the very high cooling rates. Of course exposing the sample directly to liquid nitrogen in this manner raises questions of potential contamination from the cryogen. Other approaches, such as the Cryologic Vitrification Method, still use an open device at the stage of vitrification but cool the sample by touching on a liquid nitrogen cooled aluminium block. This means that the sample is not directly exposed to the liquid nitrogen and the block avoids the Leidenfrost effect. Alternative approaches use closed straws. These avoid the contamination issues but at the expense of the cooling rate. By definition, the vitrification stage of the process is difficult to measure, monitor or document, so validation and on-going quality control are qualitative exercises only.

3.6 Stirling engines

Originally conceived in 1816 by the Reverend Stirling, the Stirling engine converts heat energy into mechanical work. The principal also works the other way round to convert mechanical energy to heat, when the Stirling engine forms a heat pump able to move heat 'uphill' from a cold place to a warmer one. This gives the Stirling engine an application as a refrigeration unit.

Most refrigerators operate on the Rankine cycle which depends on refrigerants existing with appropriate boiling points. Triple stage Rankine machines are at the limit of the technology and achieve roughly -140 °C. Although the Stirling cycle is less efficient than Rankine cycle machines, it is capable of cooling to lower temperatures and therefore comes into its own below -140 °C; miniature cryo-coolers based on Stirling engines are now quite common. Due to relative inefficiency, these Stirling based cryo-coolers can normally freeze only quite small samples of a few tens of grams maximum and cannot compete with liquid nitrogen powered machines for cooling capacity. On the other hand, they excel in clean rooms where it is not possible to obtain a supply of liquid nitrogen and it is only desired to freeze very small samples.

3.7 Liquidus tracking equipment

Because liquidus tracking is a relatively novel technique, there is little choice of equipment to assist with research into its use. Planer plc do manufacture a Liquidus Tracking controller that can be used for research into this approach. The equipment comprises a conventional slow-rate chamber coupled with a liquidus tracking controller and two peristaltic pumps.

The controller cools the sample in a similar manner to the conventional slow-rate freezing process. The cooling profile is typically a simple linear ramp. During the cooling of the sample, the controller monitors the current chamber temperature and adjusts the speeds of the two pumps to dynamically alter the concentration of cryoprotectant surrounding the samples. In the ideal process the concentration of cryoprotectant is maintained just above the liquidus curve. As the temperature decreases, the concentration of cryoprotectant is therefore increased. However, as the temperature of the sample decreases, the toxicity of the cryoprotectant decreases and this allows the sample to tolerate the ever increasing concentrations; see figure 1.
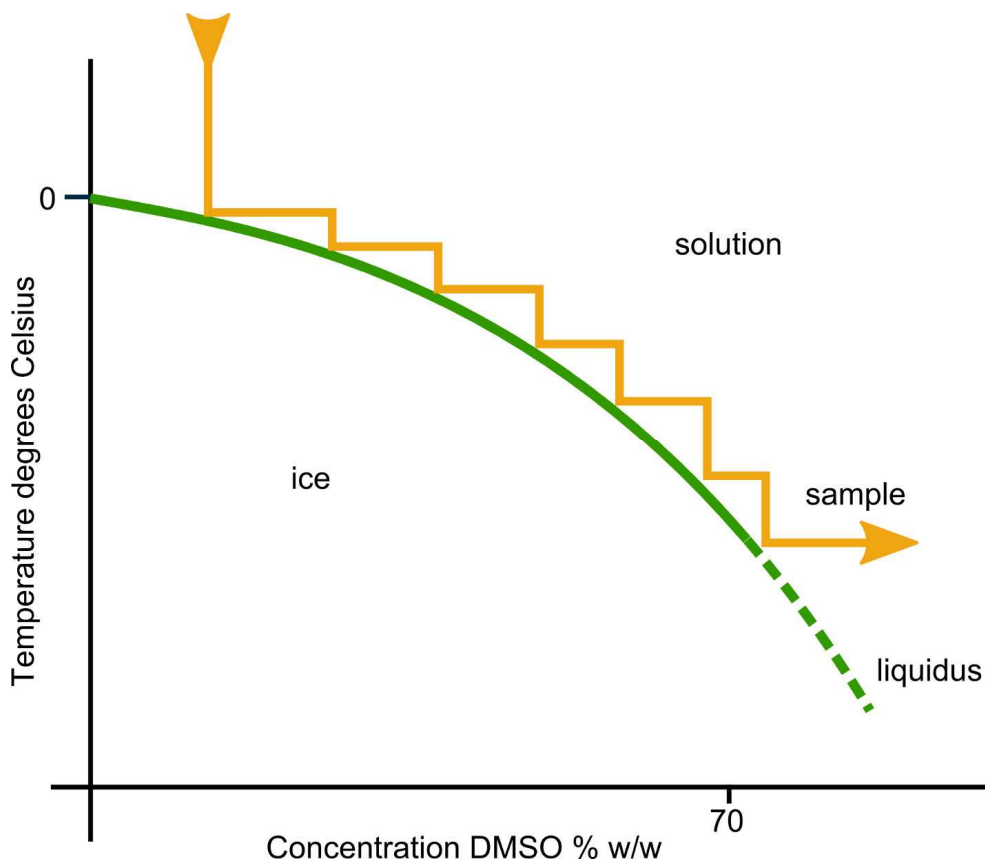
Fig. 1. Concentration of sample tracking liquidus

Two specific requirements of this process are the rather large volumes of cryoprotectant required and the need to ensure good mixing around the sample. The Planer Liquidus Tracker supports two modes of operation each with its own advantages and disadvantages; these are the single solution and dual solution modes.

In the dual solution mode, shown in figure 2, a solution containing a high concentration of CPA (typically 72 % w/w DMSO plus isotonic salts) and a solution containing only isotonic salts (nominal 0 % solution) are used. Each solution is pumped through a mixing junction and a heat exchanger into the sample container and thence into a waste collection container. The relative speeds of the pumps are continuously adjusted via a computer program to deliver the correct concentration to the sample. The pump speeds are adjusted to maintain a constant flow rate through the sample container. The dual solution system requires a small volume surrounding the sample so that the incoming, premixed solution is able to displace the existing solution completely as it flows through the container. The total volume of cryoprotectant can be quite large; for example, a run from 0 °C to -70 °C at 0.3 °C/minute requires a total volume of solution equal to 233 times the sample container volume. This

method is suitable for use with small sample containers and has been used for discs of ovine articular cartilage (see Wang et al., 2007).

Simplified view of pump system. Vents and relief paths have been omitted for clarity.
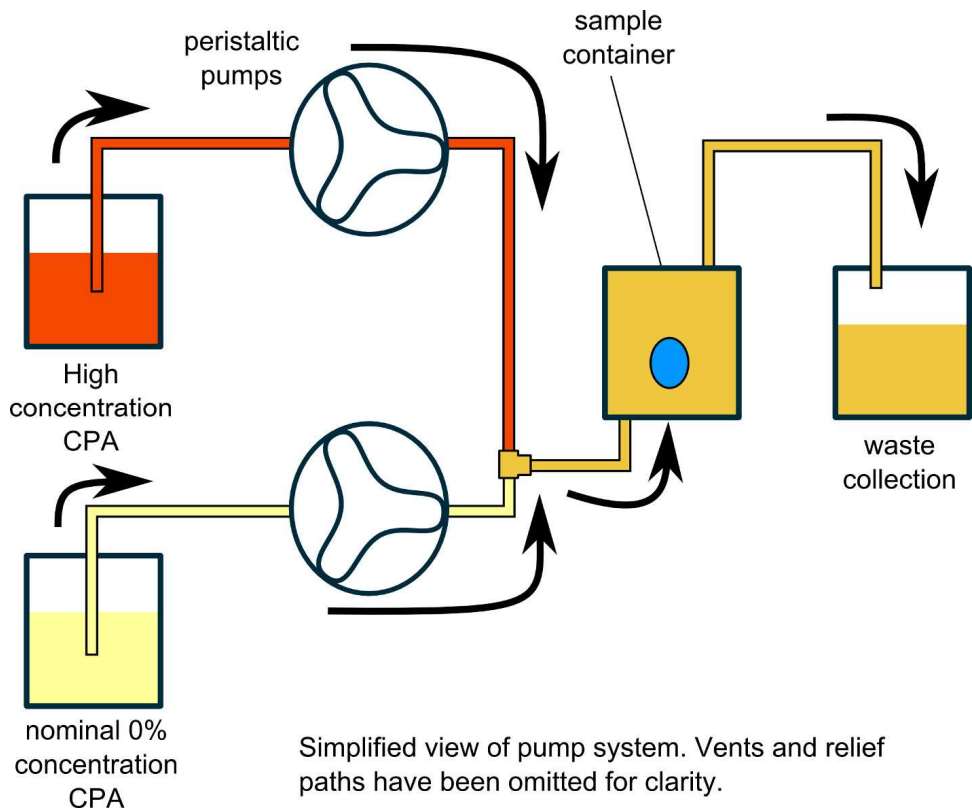
Fig. 2. Dual solution mode.

The single solution mode is more suitable for use with larger samples. It is illustrated in figure 3. Here a highly concentrated solution is cooled and delivered to the sample container. This increases the concentration of the CPA solution surrounding the sample. To maintain a constant volume within the container, the second pump extracts the excess solution from the container. This technique is suitable for larger samples as it reduces the total volume of cryoprotectant required. For a sample container volume of Vs, a cryoprotectant concentration of Ksol and a target concentration of Kt, the volume of cryoprotectant V can be calculated from this equation.

$$V = Vs.ln(Ksol / (Ksol - kt)) \tag{1}$$

For a sample container volume of 50 ml, depending on the actual values of Ksol and Kt this approach could require less than 100 ml of concentrated DMSO solution. Because the incoming solution has to be thoroughly mixed within the container, additional stirring equipment running at cryogenic temperatures is required. This results in a mechanically more complex arrangement than the dual solution approach.
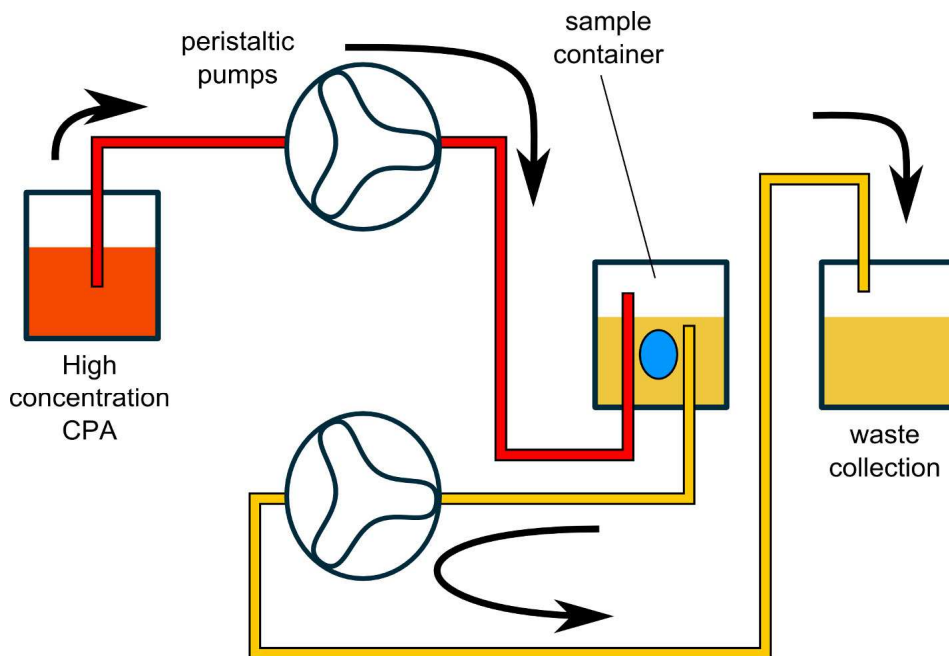
Simplified view of pump system. Vents and relief paths have been omitted for clarity.

Fig. 3. Single solution mode.

3.8 Examples of equipment used

The BioCool Controlled Rate Freezer from FTS Systems/SP Scientific is a mechanically refrigerated bath with temperature control to -40 °C or -80 °C. The fluid in the 2 litre bath provides temperature stability and dispersion of the heat of fusion without a concomitant temperature rise. Courtesy SP Scientific and Gary Gold Photography.

BioCool™ Controlled Rate Freezer

The Asymptote EF600, a unique liquid nitrogen free, controlled-rate freezer, is electrically powered by a Stirling Cycle Cryocooler rather than liquid nitrogen. This allows the freezer to be used where liquid nitrogen is in short supply, where extra high air quality is needed, or where there is a risk of LN2 contamination to samples.

Asymptote liquid nitrogen free controlled-rate freezer

The Planer Kryo 360 cell freezer controls down to a -180 °C end temperature to ensure sample integrity during transfer to storage. Fully programmable, it allows the use of protocols associated with the most advanced cryopreservation techniques and is widely used in laboratories around the world.

Planer Kryo 360 programmable cell freezer

The Crysalys controlled rate freezer: programmes, time and temperatures may be entered via a touch screen with up to 100,000 cycles held on the onboard SD card; the data can be retrieved by any PC or Mac computer. A battery back up operates the system for 3 hours; its portability and 3.2 kg weight make it especially suited to veterinary purposes.
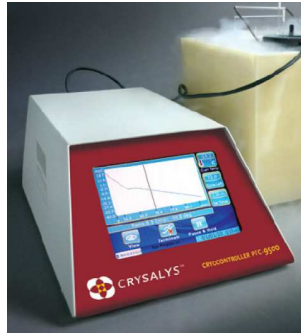
Crysalys controlled rate freezer

The Gemini Tinytag View 2 data logger, when used with a specially designed probe, is used for temperature monitoring in cryogenic environments down to -200 °C.

Tinytag View 2 data logger

The CoolCell is an alcohol-free cell freezing container which provides a reproducible cooling rate of 1 °C/minute when placed in a -80 °C freezer. No alcohol is required to control the freeze rate as the design and materials of the CoolCell ensure precise and uniform heat removal from cryovials.

CoolCell®, an alcohol-free cell freezing container

The Planer ShipsLog is a datalogger specifically designed for vapour shippers, which maintains a downloadable temperature history of samples during transit.

ShipsLog datalogger for vapour shippers

The Liquidus Tracker is a new controlled vitrifier for cryopreservation of samples using the liquidus tracking technique. This approach may have uses in vitrifying larger samples and those which are currently difficult to cryopreserve.

Liquidus Tracker controlled vitrifier for cryopreservation

4. Acknowledgement

Additional material can be found in 'Cryopreservation and Freeze-Drying Protocols'; Day, J.; Stacey, G. (Eds.); 2nd ed., 2007 Humana Press Cryopreservation of Animal and Human Cell Lines; See

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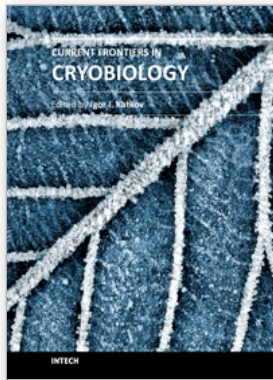
Current Frontiers in Cryobiology

Edited by Prof. Igor Katkov

Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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